Issued March 11, 1913.

U. S. DEPARTMENT OF AGRICULTURE.

BUREAU OF PLANT INDUSTRY—BULLETIN NO. 270.

B. T. GALLOWAY, *Chief of Bureau.*

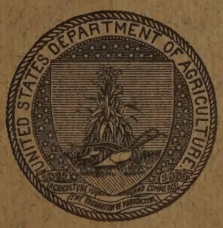
CONTRIBUTIONS TO THE STUDY OF MAIZE DETERIORATION.

BIOCHEMICAL AND TOXICOLOGICAL INVESTIGATIONS OF PENICILLIUM PUBERULUM AND PENICILLIUM STOLONIFERUM.

BY

CARL L. ALSBERG AND OTIS F. BLACK,

Chemical Biologists, Drug-Plant, Poisonous-Plant, Physiological, and Fermentation Investigations.



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BUREAU OF PLANT INDUSTRY.

Chief of Bureau, BEVERLY T. GALLOWAY.
Assistant Chief of Bureau, WILLIAM A. TAYLOR.
Editor, J. E. ROCKWELL.
Chief Clerk, JAMES E. JONES.

DRUG-PLANT, POISONOUS-PLANT, PHYSIOLOGICAL, AND FERMENTATION INVESTIGATIONS.

SCIENTIFIC STAFF.

Rodney H. True, *Physiologist in Charge.*

A. B. Clawson, Heinrich Hasselbring, C. Dwight Marsh, W. W. Stockberger, and Walter Van Fleet, *Physiologists.*
Carl L. Alsberg, H. H. Bartlett, Otis F. Black, H. H. Bunzel, Frank Rabak, and A. F. Sievers, *Chemical Biologists.*
W. W. Eggleston, *Assistant Botanist.*
S. C. Hood, G. F. Mitchell, James Thompson, and T. B. Young, *Scientific Assistants.*
Hadleigh Marsh, *Assistant.*
G. A. Russell, *Special Agent.*

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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Plant Industry,
Office of the Chief,
Washington, D. C., September 25, 1912.

Sir: I have the honor to transmit herewith and to recommend for publication as Bulletin No. 270 of the series of this Bureau the accompanying manuscript entitled "Contributions to the Study of Maize Deterioration. Biochemical and Toxicological Investigations of Penicillium Puberulum and Penicillium Stoloniferum." The paper was prepared by Dr. Carl L. Alsberg and Mr. Otis F. Black, Chemical Biologists in the Office of Drug-Plant, Poisonous-Plant, Physiological, and Fermentation Investigations, and has been submitted by Dr. R. H. True, Physiologist in Charge, with a view to its publication.

The results of technical laboratory studies of organisms occurring in deteriorated maize, (1) *Penicillium puberulum* Bainier and (2) *Penicillium stoloniferum* Thom, are here presented, demonstrating that these organisms have specific physiological properties. One of these molds is shown to develop toxic substances in maize. Owing to the serious problems now grouping themselves about this important American farm crop, it is believed that the results of this investigation constitute a timely contribution to our information on the subject of the deterioration of maize.

Respectfully,

B. T. Galloway,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

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INTRODUCTION.

Whether molds or the products of their growth have an injurious effect on animals is a question which has not yet been conclusively settled. The literature contains many records of alleged intoxications due to these fungi. Certain diseases of men and domesticated animals have been attributed to this cause. Though the solution of this problem is obviously urgent, few serious attempts have been made to identify chemically the alleged toxic substances. The present paper is such a chemical study. Incidental observations on the metabolism of molds have been made and have been recorded because they have a general biological interest and because they may prove useful in characterizing different species physiologically.

The difference of opinion concerning the toxicity of Penicillium is probably due not merely to the fact that the earlier investigators studied accidental mixtures of organisms under varying and undefined conditions,[1] but also that complex substrata like corn, wheat, and bread were used for the growth of the organisms. Consequently it is impossible to know whether any of the different substances found were derived from the substratum or were produced by the

[1] Lombroso, Cesare, and Dupré, Francesco. Indagini chimiche, fisiologiche e terapeutiche sul mais guasto. Reale Istituto Lombardo di Scienze e Lettere, Rendiconti, s. 2, v. 5, p. 882–884, 1872.

——— and Erba, Carlo. Sulle sostanze stricniche e narcotiche del mais guasto. Idem, s. 2, v. 9, p. 133–147, 1876.

——— Sull' alcaloide del mais guasto. Idem, s. 2, v. 9, p. 433–436, 1876.

——— I veleni del mais e la pellagra. Idem, s. 2, v. 9, p. 182–186, 1876.

——— I veleni del mais e la loro applicazione all' igiene ed alla terapia. Rivista Clinica di Bologna, s. 2, ann. 7, p. 109–112, 1877.

Brugnatelli, T., and Zenoni, E. Di un alcaloide che si trova nella melica guasta e nel pane di mais ammuffito. Reale Istituto Lombardo di Scienze e Lettere, Rendiconti, s. 2, v. 9, p. 293–297, 1876.

Pelloggio, Pietro. Materia reagente quale alcaloide, trovata nell' estratto del maiz guasto preparato dall' erba. Idem, s. 2, v. 9, p. 118–121, 1876.

Selmi, Antonio. Delle alterazioni alle quali soggiace il granturco (Zea mais) e specialmente di quello che ingenera la pellagra. Atti della R. Accademia dei Lincei, s. 3, Memorie della Classe di Scienze Fisiche, Matematiche e Naturali, v. 1, dispensa 2, p. 1099–1141, 1877.

Husemann, Th. Ueber einige Producte des gefaulten Mais. Ein Beitrag zur Lehre von den Fäulnissgifte. Nach Versuchen von Dr. Roberto Cortez aus Tumaco in Columbien. Archiv für Experimentelle Pathologie und Pharmakologie, Bd. 9, p. 226–228, 1878.

Monselise, G. Ricerche chimico-tossicologiche intorno ad alcuni campioni di mais per lo studio della pellagra, Mondovi, 1881, 58 p. (Cited by Gosio.)

organisms. Thus Selmi [1] thought acrolein or a condensation product of acrolein with ammonia was produced, while Lombroso and Dupré,[1] Lombroso and Erba,[1] Brugnatelli and Zenoni,[1] Pelloggio,[1] and Coeytaux [2] found alkaloids. Though some of these investigators examined the maize or other material employed as substratum, before the development of the organisms, only Lussana and Ciotto [3] give sufficient details to inspire confidence in the adequacy of the controls. They found alkaloids in both moldy and sound maize and wheat. In the investigation of maize in progress in this laboratory, of which this bulletin is in part a report, cholin, betain, and bases unidentified as yet have been isolated from sound maize.

The first investigator to use pure cultures in a simple culture medium was Gosio,[4] who used Raulin's solution. Under these conditions Penicillium endowed the culture medium with the power to react like a phenol with weak aqueous ferric-chlorid solutions. A similar observation on *Aspergillus niger* had been made many years before by Raulin,[5] who found that in the absence of iron this fungus has a similar effect on the culture medium. Raulin attributed the ferric-chlorid reaction present under these conditions to the formation of sulphocyanid. This observation was apparently unknown to Gosio. Recently Javillier and Sauton [5] have confirmed Raulin's observations, but have doubted that the reaction was due to sulphocyanid. They found, furthermore, that the reaction fails when the organism is grown in the absence of both iron and zinc. Moreover, Raciborski [6] also obtained the ferric-chlorid reaction with a number of fungi. He concluded that molds may secrete a variety of aromatic substances, for the culture medium may give Millon's reaction and the diazo reaction of Griess. He also found that substances reducing Fehling's solution, ammoniacal silver solution, and ammonium vanadate were formed. He regarded them all as probably products of the protein metabolism of the fungus.

Gosio found further that by administering the culture medium to mice, to rats, to guinea pigs, to rabbits, to cats, or to dogs, symptoms resembling phenol poisoning could be produced. The culture media

[1] Op. cit.

[2] Coeytaux, A. Notice sur l'huile et la teinture de mais gâté. Schweizerische Wochenschrift für Pharmacie, Jahrg. 18, p. 153–156, 1880.

[3] Lussana, Filippo, and Ciotto, Francesco. Su gli alcaloidi del mais guasto. Gazzetta Medica Italiana Lombardia, v. 43 (s. 8, t. 5), p. 522–523, 1883; v. 44 (s. 8, t. 6) p. 82–86, 95–97, 105–106, 122–123, 126–130, 149–150 167–168, 173–179, 196, 243–247, 263–266, 273–276, 283–287, 294–296, 1884.

[4] Gosio, B. Ricerche batteriologiche e chimiche sulle alterazioni del mais. Rivista d'Igiene e Sanità Pubblica, ann. 7, p. 825–849, 869–888, 1896.

[5] Cited by Javillier, M., and Sauton, B. Le fer est-il indispensable à la formation des conidies de l'Aspergillus niger? Comptes Rendus de l'Académie des Sciences [Paris], t. 153, p. 1177–1180, 1911.

[6] Raciborski, M. Über die Assimilation der Stickstoffverbindungen durch Pilze. Bulletin International de l'Académie des Sciences de Cracovie. Classe des Sciences Mathématiques et Naturelles, ann. 1906, p. 733–770, 1907.

presented other properties characteristic of phenols. Hence, Gosio concluded that phenols were produced by the molds and that the toxicity of the culture media was due to their presence. By means of ether he even succeeded in isolating from the culture medium a small quantity of a crystalline substance giving the ferric-chlorid color reaction. The substance was slightly soluble in cold water, though freely soluble in hot water and in most organic solvents. It failed to react with Fehling's solution or with phenylhydrazin. It contained 62.2 per cent carbon, 6.34 per cent hydrogen, and 28.45 per cent oxygen, corresponding to the empirical formula $C_9H_{10}O_3$. In alcoholic solution ferric chlorid produced an intense blue coloration. In spite of the high melting point, 143° to 144° C., and the failure of Millon's reaction, Gosio regarded the substance as probably ρ-hydro-cumaric acid. Moreover, he presented evidence for the existence in the culture medium of other unisolated phenolic substances. As a result of these researches Gosio proposed ferric chlorid as a reagent for the detection of moldy maize, but stated that its usefulness might be limited to the detection of the molds producing phenols.[1] These discoveries, in the main, were confirmed by others.[2]

Unfortunately, none of the investigations on green Penicillium can be repeated because the organism used was inadequately described. Undoubtedly the different investigators employed different species, for nearly all of them recognized the fact that the quantities of Gosio's toxic phenolic substances varied for different strains. None of them attributed these variations to the use of distinct species, though Ceni and Besta[3] did distinguish between toxic and nontoxic forms without, however, adequately differentiating them.

Di Pietro[4] first showed toxicity to be limited to certain distinct species of Penicillium. From moldy maize he isolated *Penicillium brevicaule, P. candidum,* and *P. glaucum;* but stated that "*P. glaucum*" did not mean *P. glaucum* Link, because six quite distinct green species were isolated, none of which is described in Saccardo's Sylloge. Of

[1] Black, O. F., and Alsberg, C. L. The determination of the deterioration of maize, with incidental reference to pellagra. U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 199, p. 27, 1910.

[2] Gosio, B., and Ferrati, E. Sull' azione fisiologica dei veleni del mais invaso da alcuni ifomiceti. Rivista d'Igiene e Sanità Pubblica, ann. 7, p. 961–981, 1896.

Antonini, G., and Ferrati, E. Sulla tossicità del mais invaso da "Penicillium glaucum." Archivio di Psichiatria, Scienze Penali ed Antropologia Criminale, v. 24 (s. 2, v. 8), p. 581–585, 1903.

Ceni, Carlo. Le proprietà tossiche di alcuni ifomiceti in rapporto colle stagioni e col ciclo annale dell'endemia pellagrosa. Comunicazione preliminare. Rivista Pellagrologica Italiana, v. 4, p. 89.

——— Idem. Seconda nota preventiva. Comunicazione fatta al III Congresso della Società Italiana di Patologia tentosa in Roma, 26-28 Aprile, 1905. Rivista Pellagrologica Italiana, v. 5, p. 184.

[3] Ceni, Carlo, and Besta, Carlo. I Penicilli nell' etiologia e patogenesi della pellagra. Rivista Sperimentale di Freniatria e Medicina Legale della Alienazioni Mentali, v. 29, p. 741–815, 1903.

——— Die pathogenen Eigenschaften des Aspergillus niger mit Bezug auf die Genese der Pellagra. Beiträge zur Pathologischen Anatomie und zur Allgemeinen Pathologie, Bd. 37, p, 573–589, 1905.

[4] Di Pietro, Melchiorre. Sui veleni di alcune muffe. Annali d'Igiene Sperimentale, v. 12 (n. s.), p. 314–365, 1902.

——— Intorno al "Penicillio tossico." Rivista Pellagrologica Italiana, v. 3, p. 221, 1903.

all the organisms isolated from maize by Di Pietro only one of the six species of "*P. glaucum*" proved to be toxic. The published description of the organism [1] was not accessible in Washington.

Gosio [2] replied to these papers of Di Pietro, stating that he had previously pointed out [3] that different strains vary greatly in their power to produce toxic substances. Though he studied Di Pietro's organism he refused to acknowledge that Di Pietro's observations had any further significance and was inclined to think that *Penicillium glaucum*, like pathogenic bacteria, varies in virulence.

Di Pietro believed that the toxic principle was a glucosid,[4] although his reasons were not clearly stated, for this toxic principle was not obtained in a state of purity. All that showed glucosidic properties was the reduction of Fehling's solution by the extracts and the diminution of toxicity by heating with hydrochloric acid. Though Gosio's substances were excreted into the medium and those of Di Pietro were confined to the spores, both substances gave a ferric-chlorid color reaction.

Sturli [5] also found that an organism isolated from polenta or maize mush from Bukowina did not render the medium toxic, while the mycelium was very toxic. He believed it premature to draw any conclusions concerning the chemical properties of the toxic substances, calling the toxic material "indifferent substances."

Possibly the descriptions of all of the green species of Penicillium used in previous investigations are inadequate because the medical bacteriologist is usually insufficiently acquainted with molds.[6] The identification of the species can be intrusted only to the skilled mycologist.[7] One of the consequences of the study of molds by mycologists has been that Thom has found it necessary to disregard the species *Penicillium glaucum* Link, because Link's description is applicable to many forms.[8] Hence the identity of many of the molds used hitherto in toxicological investigations can no longer be established. Di Pietro was therefore right in his belief that a

[1] Di Pietro, Melchiorre. Studio morfologico e biologico sul Penicillium glaucum (var. tossica) Teramo. 1904.

[2] Gosio, B. Per l'etiologia della pellagra. Rivista Pellagrologica Italiana, v. 3, p. 177, 1903.

[3] Gosio, B. Ricerche batteriologiche e chimiche sulle alterazioni del mais. Rivista d'Igiene e Sanità Pubblica, ann. 7, p. 825–849, 869–888, 1896.

[4] Di Pietro, Melchiorre. Glucosidi di elevato potere tossico trovato nelle spore del Penicillium glaucum. Rivista Pellagrologica Italiana, v. 2, p. 63, 1902.

[5] Sturli, Adriano. Ueber ein in Schimmelpilzen (Penicillium glaucum) vorkommendes Gift. Wiener Klinische Wochenschrift, Jahrg., p. 711–714, 1908.

[6] Tiraboschi, C. Studi sugli ifomiceti parassiti del granturco guasto. Atti del Terzo Congresso Pellagrologico Italiano, Milano, 24–25–26, Septembre, 1906, p. 126. 1907.

[7] Nikitinsky, Jacob. Über die Beeinflussung der Entwicklung einiger Schimmelpilze durch ihre Stoffwechselprodukte. Jahrbücher für Wissenschaftliche Botanik, Bd. 40, p. 3, footnote, 1904.

Wehmer, C. Notiz über Rhizopus-Arten. Berichte der Deutschen Botanischen Gesellschaft, Bd. 28, p. 547–549, 1911.

[8] Thom, Charles. Cultural studies of species of Penicillium. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 118, 1910.

number of green species exist. Moreover Dox[1] has shown that biochemically the different species vary and has asserted that many of the conflicting statements in the literature, especially those concerning the proteolytic enzyms, may be explained on the assumption that different organisms were used under the same name. Nikitinsky[2] also suspected that *Penicillium glaucum* and *P. griseum* comprised a number of physiological varieties because of their varying power to form or to withstand acid.

For this investigation the genus Penicillium was chosen, because it is well systematized in the recent monograph of Thom.[3] Five species inhabiting spoiled maize in the United States were obtained from Dr. Thom and cultivated separately by Dr. Erwin F. Smith. Without such help this investigation could hardly have been undertaken. Seventy-five to one hundred grams of air-dry, white corn meal were sterilized, usually in the autoclave, in 1-liter Erlenmeyer flasks, with enough water to form a layer of the mixture over the bottom about 3.75 centimeters thick. These flasks were observed for several days, to detect inadequate sterilization, when the sterile flasks were inoculated by Dr. Smith with spores of fresh cultures. The cultures were allowed to develop at room temperature in diffused light for 34 days, from April 12 to May 16, 1910. All developed spores.

To determine toxicity each culture was digested 16 hours with 500 cubic centimeters of 95 per cent alcohol. The extract was filtered and all alcohol from 50 cubic centimeters, corresponding to 6 to 10 grams of meal, evaporated on the steam bath. The residue was filtered, yielding usually about 1 cubic centimeter of an acid solution. One-half of this filtrate was then injected subcutaneously into mice weighing 20 grams each. The extract from one of the cultures caused death in about 10 hours; another in 7 hours; the others had no harmful effect. Neither the convulsions nor the spasms described by Gosio and Ferrati[4] were observed. The mice seemed merely very sick and died quietly. When the dosage was larger, initial convulsions with paralysis of the fore limbs were produced. These symptoms soon passed away and the mice died as did those that had received smaller dosages. The fatal dose for both cultures was more than ten times that of Gosio's cultures, so that neither is likely to have been identical with his culture.

As a control, a culture flask containing corn-meal mush in the same quantity was left uninoculated. After standing sterile during the

[1] Dox, A. W. The intracellular enzyms of Penicillium and Aspergillus, with special reference to those of Penicillium camemberti. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 120, p. 36, 1910.

[2] Nikitinsky, J. Op. cit.

[3] Thom, Charles. Op. cit.

[4] Gosio, B., and Ferrati, E. Sull' azione fisiologica dei veleni del mais invaso da alcuni ifomiceti. Rivista d'Igiene e Sanità Pubblica, ann. 7, p. 961-981, 1896.

growth of the organisms in the other flasks it was, simultaneously with them, extracted by the same proceeding and tested in the same way for toxicity. It did not produce any symptoms in the mice. Consequently, the effects observed were the result of the growth of the molds.

Because the toxic substances are believed by Gosio to be identical with those giving color reactions with ferric chlorid the cultures were tested with this reagent. Fifty cubic centimeters of the alcoholic extracts were neutralized and evaporated nearly to dryness on the steam bath. The residues were then extracted with water weakly acidulated with hydrochloric acid and filtered. The clear filtrate was extracted with a relatively large volume of ether and the ethereal extract evaporated to dryness. The residue from the ether was extracted with water before testing with a very weak solution of ferric chlorid. In one case a weak but distinctly reddish brown color was obtained. One culture gave no color. Two gave a slight brown coloration. Another culture, after the evaporation of the alcohol, gave a brick-red, watery extract, which, on neutralization, turned to a deep-claret color. Therefore, in this culture, it was impossible by this method to detect phenols, if present. The organism producing this dye is the one referred to by Thom [1] as having been found by Prof. F. D. Heald on maize and as being either identical with or very closely related to *Penicillium purpurogenum* O. Stoll. It has been possible to isolate the dyestuff and its description is reserved for a future publication.

The culture which gave on extraction the brownish red coloration with ferric chlorid was the one that killed the mice in 10 hours. It was identified as *Penicillium puberulum* Bainier, and a detailed study of this species is here given.

PENICILLIUM PUBERULUM.

Penicillium puberulum Bainier was originally isolated from corn by Prof. F. D. Heald, then of Lincoln, Nebr., now of Austin, Tex. The culture obtained from Dr. Thom is No. 45 of his collection, and had been grown on a 3 per cent sugar decoction of beans. Dr. Thom states that if it is not identical with *P. puberulum* of Bainier [2] it is very closely related to it. He has very kindly furnished the following description:

Colonies in Czapek's solution agar, dull bluish green to green, velvety, spreading slowly upon the substratum, zonate at the margin in older colonies, with an outer band of submerged mycelium; mycelium yellowish or greenish to tan below (as viewed from below); agar uncolored; odor weak but noticeable; conidiophores

[1] Thom, Charles. Op. cit., p. 36.

[2] Bainier, G. Sur dix espèces nouvelles de Penicillium et sur le genre G. graphiopsis. Bulletin, Société Mycologique de France, t. 23, 1907, p. 16. 1908.

FLASK SHOWING TWELVE DAYS' GROWTH OF PENICILLIUM PUBERULUM ON RAULIN'S MEDIUM.

mostly arising separately from the submerged hyphæ, short—up to 100 μ long by 3.5 μ diameter, crowded, with walls more or less rough with delicate warts or granules or smooth; conidial fructifications becoming more or less loosely columnar masses 20 to 30 μ long and consisting of the main conidiophore, a primary branch or verticil of branches, secondary verticils of branchlets, all swollen or enlarged at apex, bearing verticils of conidiferous cells 8.5 by 2 to 2.5 μ, producing very long chains of conidia; conidia elliptical to globose, 3 to 3.5 μ diameter when ripe, smooth, swelling in germination to 5 to 8 μ and germinating by one or two tubes 3 μ in diameter—when two tubes are present these are separated by 180°—appear at the points of contact of the conidia in the chains (where the walls are thinner); colonies liquefy simple gelatin, producing a trace of brown color in the liquid and an alkaline reaction to litmus, and become dark or smoky in color in media without sugar; with lactose gelatin no acid is produced; in rich substrata under humid conditions colonies become overgrown with white floccose sterile mycelium after the conidial areas are matured; colonies grown readily in all common media. Collected by Prof. F. D. Heald in Nebraska upon decaying Indian corn (*Zea mays*).

Note.—In measuring conidiophores I measure from the point of origin at the surface of the substratum to the lowest branch of the fruit. Simple gelatin here means 15 per cent of gelatin in distilled water.

Plate I shows a culture of *Penicillium puberulum* Bainier after 12 days' growth on Raulin's medium.

In investigating the toxic properties of this mold, the injurious effects were found due not to the acidity [1] of the extracts of the culture, as prepared above, but to some specific substance. This is shown in the following experiment:

Fifty cubic centimeters of the alcoholic extract of the corn-meal cultures were carefully neutralized with sodium carbonate before concentration. The solution thus obtained was not toxic. As loss of toxicity might be due either to neutralization of acid or to destruction of the toxic substance by heat in neutral solution, fresh extracts were tested by injecting the ethereal extract neutralized after concentrating. The mice died as before, showing that the toxicity was due to some toxic material stable only in acid solution.

PENICILLIC ACID FROM PENICILLIUM PUBERULUM.

Cultures of *Penicillium puberulum* Bainier were grown about a month on Raulin's medium. At the end of this period the medium was still acid, but far less toxic than the extracts prepared from the cultures on corn-meal mush. However, the medium gave the color reaction with ferric chlorid. Extracts of the mycelium were neither toxic nor did they give the ferric-chlorid reaction. The substance responsible for this reaction did not occur in the mycelium. It was extracted from the medium with chloroform and crystallized on concentration of the solvent. Recrystallized from hot water it forms large, transparent, biaxial, monoclinic or triclinic, rhombic crystals

[1] Black, O. F., and Alsberg, C. L. The determination of the deterioration of maize, with incidental reference to pellagra. U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 199, p. 30, 1910.

with good cleavage normal to an axis. The substance burns without ash, contains no nitrogen, and is not optically active; but it is acid to litmus and phenolphthalein and decomposes carbonates at ordinary temperatures.

For purposes of analysis the material was purified with bone black and twice recrystallized from hot water. Recrystallization from alcohol, water, ether, benzol, or chloroform and precipitation from ethereal solution by petroleum ether failed to remove traces of a yellowish color or to change the melting point.

The crystals from water have a melting point of 64° to 65° C., uncorrected, and effloresce rapidly in the air. The anhydrous substance is a white powder, melting sharply at 86° to 87° C., uncorrected, without decomposition. The water of crystallization was determined by pressing the crystals dry between filter paper, weighing rapidly, and drying to constant weight at room temperature under diminished pressure, with sulphuric acid as dehydrating agent. Heated to 50° to 60° C. the material suffered no further loss in weight. The other analyses made were the determinations of the content of carbon and hydrogen, of the magnesium content of the salt, and the titration of the free acid with standard alkali. The last two determinations furnished data for the calculation of the molecular weight. The substance was dried for these analyses in the manner above given. The analytical data follow:

TABLE I.—*Analyses of penicillic acid.*

Weight of substance (grams).	CO_2 (grams).	H_2O (grams).	C (per cent).	H (per cent).	Water of crystallization.		$MgSO_4$ (grams).	Mg (per cent).	N/10KOH (c. c).
					Grams.	Per cent.			
0.2268 (anhydrous substance)	0.4649	0.1165	55.90	5.71					
0.2017 (anhydrous substance)	.4135	.1081	55.91	5.68					
0.2215 (anhydrous substance)									12.97
0.3460 (crystals from water)					0.0577	16.6			
0.2664 (crystals from water)					.0475	17.8			
0.2238 (anhydrous magnesium salt)							0.0750	6.78	
0.1923 (anhydrous magnesium salt)							.0639	6.72	
Average			55.90	5.69		17.2		6.75	

Calculated for $C_8H_{10}O_4$: carbon, 56.6 per cent; hydrogen, 5.89 per cent.
Found ..... carbon, 55.90 per cent; hydrogen, 5.69 per cent.
Calculated for $C_8H_{10}O_4$, $2H_2O$: water, 17.48 per cent.
Found ..... water, 17.20 per cent.

A molecular weight determination by the elevation of the boiling point in chloroform solution gave the results shown in Table II.

TABLE II.—*Ebullioscopic determination of molecular weight of penicillic acid.*

Weight of substance (grams).	Weight of solvent (grams).	Rise of boiling point (°C.).	Molecular weight.
0.1802	26.24	0.170	148
.2091	26.24	.191	153
.2349	26.24	.177	177
Average			162

Molecular weight calculated for $C_8H_{10}O_4$	170
Molecular weight found from titration	170.4
Molecular weight found from magnesium content of salt	168.3
Molecular weight found by boiling-point elevation	162

From the data at hand the formula $C_8H_{10}O_4$ may be provisionally assigned to this substance. It has not been possible to identify it with any known substance, nor has it as yet been possible to determine its constitution. Tentatively, the name penicillic acid is suggested for it.

Penicillic acid is soluble in alcohol, ether, benzol, and chloroform, but insoluble in petroleum ether. In cold water it is soluble in the proportion of about 2 to 100, but readily soluble in hot water. From its aqueous solutions it can be extracted most readily by chloroform. Olive oil dissolves it but slightly and does not extract it from aqueous solution. It decomposes carbonates. It has a somewhat salty, slightly bitter-sweet taste, and is somewhat irritating to mucous membranes. It produces a rather persistent sensation of burning at the fauces. It absorbs bromin without forming an insoluble compound; reduces Fehling's solution when heated, and ammoniacal silver at room temperature. It is fairly resistant to acids, for it may be heated with weak acid without undergoing decomposition, and it is not charred by concentrated sulphuric acid. It is, however, very sensitive to alkali. Fixed alkalis turn the solution yellow. Dilute ammonia gradually converts it into a deep-red dye, resembling that obtained from orcin by ammonia. The color with ammonia is so similar to that of the dyestuff obtained from *Penicillium purpurogenum*[1] that similarity in constitution between the two substances is at once suggested.

The magnesium salt forms transparent, rapidly efflorescent plates, drying to a white powder. It is very soluble in water and alcohol and is not readily precipitated from its alcoholic solution by ether.

[1] Cf. supra, p. 12.

The salt was prepared by digesting an aqueous solution of the pure substance on the water bath with magnesium carbonate, filtering, and concentrating to dryness in a desiccator. It contains about 30 per cent of water of crystallization. Because of its great solubility it was not further purified for analysis, but dried to constant weight in vacuo under sulphuric acid at room temperature. The salts of calcium, barium, sodium, ammonium, potassium, and copper are also very soluble in water. Their solutions dry down to varnishes. The silver salt is not stable. The lead salt is insoluble in water and amorphous.

Aqueous solutions of penicillic acid are not changed by very dilute ferric chlorid till after some time, when they gradually turn brownish red. Either it is not a simple phenol and must first be oxidized, or the action of the ferric chlorid is inhibited for a time by the reducing power of the substance. The reaction is not so delicate as that with ammonia. The formation of the color with ferric chlorid is very like that obtained by the oxidation of β-oxybutyric acid by means of weak peroxid of hydrogen containing traces of ferric chlorid.[1] Penicillic acid also gives the color under these conditions. The reaction carried out with the aid of peroxid of hydrogen is very much more delicate than that without the peroxid of hydrogen. Alcoholic solutions do not give a color with ferric chlorid. Aqueous solutions give no color with calcium hypochlorite. Nickel and cobalt chlorid give no color reaction.

Because of the observations of Brugnatelli and Zenoni[2] on the occurrence of strychninlike alkaloids in moldy corn, reactions characteristic for strychnin were tried. (1) A few crystals treated with concentrated sulphuric acid gave only a faint yellow color. On adding a small crystal of bichromate of potassium to the mixture a weak green color from reduced bichromate developed. The reaction was therefore negative. (2) A few crystals were treated with a little concentrated nitric acid. No color developed. On warming, a yellow color appeared, which, as the acid was evaporated, turned pale orange. After cooling, an orange-colored sticky residue remained, which when treated with ammonia water turned a little darker orange. Hence, both these tests for strychnin proved negative.

If an aqueous solution of the substance be boiled under a reflux condenser with barium hydrate, barium carbonate is the only insoluble product formed. If the solution be then distilled after acidifying, a small quantity of delicate needles with an odor akin to cumarin and vanillin may be separated from the distillate by extracting with

[1] Black, O. F. The detection and quantitative determination of β-oxybutyric acid in the urine. Journal of Biological Chemistry, v. 5, p. 207–210, 1908.

[2] Brugnatelli, T., and Zenoni, E. Di un alcaloide che si trova nella melica guasta e nel pane di mais ammuffito. Reale Istituto Lombardo di Scienze e Lettere, Rendiconti, s. 2, v. 9, p. 293–297, 1876.

ether. They gave no color with ferric chlorid. Gosio obtained extracts with an odor of cumarin from cultures of Aspergillus.[1]

The dry crystals heated with concentrated hydrochloric acid form a yellow oil, showing a tendency to crystallize when chilled. The oil is insoluble in water and with ferric chlorid gives a dark-red color at once, not weakly and gradually like the mother substance.

Lieberman's reaction produced a beautiful carmine-red coloration. Millon's reaction is negative. In performing Piria's reaction the substance when dissolved in warm, concentrated sulphuric acid turned yellow, then red; and when diluted with water gave a heavy, amorphous-red precipitate, which was not obtained crystalline from alcoholic solutions. This is evidently an insoluble sulphonic acid. It was removed by filtration and the filtrate neutralized with barium carbonate. The barium sulphate was removed and the clear filtrate tested with a little weak ferric chlorid. A dirty red color developed, quite different from the fine violet color obtained with tyrosin. It is doubtful whether this test has the significance of Piria's reaction, since a similar color is obtained after heating penicillic acid with dilute sulphuric or hydrochloric acid without subsequently neutralizing.

By the method suggested by Schotten and Baumann no crystalline benzoyl derivative was obtained. No crystalline oxime could be prepared. A small quantity of a crystalline derivative was obtained by heating with acetic anhydrid. Crystalline ethyl ester was also prepared by heating in absolute alcohol with a little dry hydrochloric acid. Heating an aqueous solution with phenylhydrazin hydrochlorid and sodium acetate caused decomposition, as shown by the evolution of gas. Lemon-yellow prisms were formed, which were recrystallized from hot alcohol until a constant melting point, 171° C., uncorrected, without decomposition, was obtained.

The determination of carbon and hydrogen in these yellow crystals by combustion gave the following results:

Weight of substance burned.	0.2103 gram.	
Weight of CO_2	.5655 gram;	C, 73.34 per cent.
Weight of H_2O	.1429 gram;	H, 7.56 per cent.

From these figures no formula could be calculated which might be derived from the original substance. It is probably a compound formed by the condensation of phenylhydrazin with a decomposition product. With methylphenylhydrazin nothing crystalline could be obtained. With an excess of nitrophenylhydrazin and acetic acid a red crystalline substance separates, which was not further studied.

Penicillic acid seems to be a substance not hitherto described. Hydroxyphenylglycolic acid would have a similar formula. It is

[1] Gosio, B. Mutamento de chimismo ifomicetico in rapporto all' alta e bassa fermentazione. Atti del Quarto Congresso Pellagrologico Italiano, Udine, 23-24-25 Septembre, 1909, p. 65. 1910.

not known and would in all probability give Millon's reaction. Certain of the lichen acids found hitherto only in lichens and the substance of Gosio cited above have similar empirical formulæ and properties. Not only have some of the lichen acids empirical formulæ resembling penicillic acid, but penicillic acid resembles them in its chemical behavior. Lichen acids are stable in acid solution, but are often decomposed by alkalis to form dyestuffs, as is also the case with penicillic acid. Some of the dyestuffs in common use, like orseille and litmus, are formed in this way. Most of them are decomposed by hot barium hydrate. Some of them are bitter and irritating, often much more so than penicillic acid. Some are derivatives of phenolic substances like orcin or divarin. The fact that penicillic acid forms a sulphonic acid insoluble in water and yields a dye when subjected to Lieberman's reaction also indicates that it contains a phenol nucleus. Lichen acids of this constitution turn red when treated with ammonia, like penicillic acid. Many of them give color reactions with ferric chlorid. The evidence thus far at hand decidedly indicates that penicillic acid may eventually prove to belong to this class of compounds.[1]

However, aliphatic formulæ are possible. The propyl esters of diacetylcarbonic, glyoxylpropionic, and acetylpyruvic acids would have very similar formulæ and, presumably, at least some of the characteristics of penicillic acid. Should penicillic acid ultimately prove to be a substance of this general type it would still in some ways resemble certain of the lichen acids; for example, vulpinic acid, which is an inner anhydrid of diphenylketipinnic acid.

If penicillic acid is related to the lichen acids its formation by the fungus is not strange, since lichens are symbiotic growths composed of fungi and algæ. These observations would then acquire biological interest, since they indicate that lichen acids are produced by the metabolism of the fungous part of the symbiotic lichen rather than by that of the alga. Tobler[2] concluded that lichen acids are solely the product of symbiosis, because a number of fungi from lichens grown separately without algæ formed no lichen acids. Since the formation of penicillic acid is so very dependent upon a number of conditions, it may well be that Tobler did not find lichen acids because he did not happen to employ the necessary environmental conditions.

The penicillic acid described in this paper is not the substance obtained by Gosio, as shown by the fact that the latter gives a dif-

[1] Hesse, O. Beitrag zur Kenntnis der Flechten und ihrer charakteristischen Bestandteile. Journal für Praktische Chemie, n. F., Bd. 83, p. 22-96, 1911.

[2] Tobler, F. Das physiologische Gleichgewicht von Pilz und Alge in den Flechten. Berichte der Deutschen Botanischen Gesellschaft, Bd. 27, p. 421-427, 1909.

—— Zur Ernährungsphysiologie der Flechten. Berichte der Deutschen Botanischen Gesellschaft, Bd. 29, p. 3-12, 1911.

ferent color with ferric chlorid, that it is less soluble in cold water, and that it has a higher melting point, properties in which it resembles lichen acids even more than penicillic acid does.

Nevertheless, the properties and formulæ of the two substances are so similar that some genetic relationship between them is very probable. This possibility gains likelihood from the fact, demonstrated by Gosio, that in addition to the substance isolated by him other similar ones were present giving different colors with ferric chlorid. Moreover, in his cultures the ferric-chlorid reaction varied both in intensity and quality of color. The extract of only 5 to 10 cubic centimeters of culture fluid of some cultures gave a reaction with ferric chlorid, while others required 50 to 100 cubic centimeters.

Evidence of the occurrence of other substances was encountered in the course of this investigation. Some of the culture fluid which had been exhausted by extraction with chloroform was dehydrated by treating with plaster of Paris. The dry powdered mixture was extracted with chloroform and the chloroform evaporated. The residue with an odor like cresol consisted of a few yellowish crystals in a reddish oil. The material was somewhat toxic.

Other evidences in the same direction consist of observations made upon corn spoiled naturally by unidentified green molds and giving positive ferric-chlorid reactions. In every sample encountered in this laboratory the reaction appeared at once, not gradually, as with penicillic acid. The few attempts made to isolate penicillic acid from some of these samples failed, although the acid could readily be isolated from corn inoculated with *Penicillium puberulum*. Undoubtedly in the samples substances other than penicillic acid were responsible for the ferric-chlorid reaction.

Another indication of the presence in the culture fluids of unidentified substances is the considerable acidity of the culture fluids. Four hundred and eighty-five cubic centimeters of a four weeks' culture required 92.15 cubic centimeters n/10 sodium hydroxid for neutralization. From 485 cubic centimeters of this culture 0.2 of a gram of penicillic acid was isolated, equivalent to 11.1 cubic centimeters of n/10 alkali. The acidity of fresh Raulin's solution is due entirely to the tartaric acid, but at the end of growth no tartaric acid could be isolated. On the assumption that all the tartaric acid had been consumed and that all the penicillic acid had been extracted, a considerable quantity of acid remained undetermined.

Furthermore, there are great differences in the metabolism of the organism used by Gosio and of *Penicillium puberulum*. The former produced acetaldehyde, a little acetic acid, glycerin, and succinic acid;[1] the latter produced no more than traces of these substances.

[1] Gosio, B. Ricerche batteriologiche e chimiche sulle alterazione del mais. Rivista d'Igiene e Sanità Pubblica, ann. 7, p. 874, 1896.

Finally, a conclusive argument is the fact that, as shown below, it has been possible to isolate in quantity from another species, *P. stoloniferum* Thom, a new acid more nearly resembling Gosio's substance than penicillic acid does. These facts do not support Paladino-Blandini's [1] suggestion that the toxic material formed by a large number of fungi is identical.

Di Pietro's toxic principle also differs from penicillic acid in its insolubility in water and in the fact that with ferric chlorid it gave a grass-green, "verda-erba," color instead of a reddish one.[2] Di Pietro [3] believed the toxic principle to be a glucosid. His evidence for this view, as above stated, seems to have been based on the fact that the reducing power of his toxic extracts was lost by boiling in acid, but was not lost in neutral solution. But this is also true of penicillic acid, though there is no question of its being a glucosid. If it contained a carbohydrate it would char with concentrated sulphuric acid and would in all probability be optically active. It is not necessary for a substance to be a glucosid in order to have reducing power. Many lichen acids, for example, have this power. In another important way Di Pietro's extracts resemble penicillic acid. He says that the "glucosid" itself gives the reaction with ferric chlorid only very weakly and gradually. This is exactly the behavior of penicillic acid. If, however, says Di Pietro, its sugar be split off by hydrolysis an aromatic acid is set free, giving the ferric-chlorid reaction at once. Penicillic acid behaves in quite the same way when heated with mineral acid. It is not necessary to assume that either substance is a glucosid. Treatment with acid might merely saponify an ester. It is not necessary to assume, as does Di Pietro, that the substance giving the color is present in three forms, as free aromatic acid, as a salt of the acid, giving the reaction only after acidifying, and as a glucosid. There are so many resemblances between Di Pietro's extracts and penicillic acid that the question involuntarily suggests itself whether his extracts were not mixtures of an unknown very toxic substance and penicillic acid or some very closely related substance. Di Pietro's "glucosid," Gosio's substance, and penicillic acid equally resemble lichen acids. It does not necessarily follow that the very toxic substance or substances of the extract were responsible for the "glucosid" or ferric-chlorid reactions.

CONDITIONS OF PENICILLIC-ACID FORMATION.

On Raulin's medium penicillic acid seems to accumulate only at very definite oxygen tensions. Both a full and a very scanty supply

[1] Paladino-Blandini, A. Tossici di ifomiceti. Archivio di Farmacologia Sperimentale e Scienze Affini, v. 5, p. 606-664, 1906.

[2] Di Pietro, Melchiorre. Intorno al "Penicillio tossico." Rivista Pellagrologica Italiana, v. 3, p. 221, 1903.

[3] Di Pietro, Melchiorre. Glucosidi di elevato potere tossico trovato nelle spore del Penicillium glaucum. Rivista Pellagrologica Italiana, v. 2, p. 63, 1902.

of air are unfavorable. When to insure good aeration the organism was grown in rectangular quart bottles, known in the trade as "Long Blakes," turned on their sides and containing about 200 cubic centimeters of medium, no penicillic acid could be found except traces in cultures less than a week old. Under these conditions [1] the exposed surface of the medium is relatively great and the aeration is good because the neck of the bottle is only about 1 centimeter above the surface. It was hoped that under these conditions the growth of mycelium would be more rapid and the yield of penicillic acid greater than in the 2-liter Erlenmeyer flasks, in which the same amount of medium offers far less surface and the carbonic acid must diffuse upward more than 20 centimeters before escaping from the tightly plugged neck of the flask. Growth was actually better in the bottles, but the yields of penicillic acid were insignificant. Perhaps the good aeration accounts for the latter phenomenon, since penicillic acid is easily oxidized and since even in the Erlenmeyer flasks the yields were poor when they were very loosely plugged with cotton. On the other hand, too poor a supply of air also prevents the formation of penicillic acid, as was shown when the supply was almost completely cut off by tying thin rubber sheeting over the necks of flasks and bottles. This deficiency of yield may be due to the fact that, even after a month, growth is scanty and without spore formation, though it is somewhat better in the bottles than in the flasks. The culture medium remained white and transparent. When the seal was removed from one of the bottles, growth and spore formation became rapid. Within 24 hours the medium turned quite dark. After six days a very small quantity of penicillic acid could be isolated—rather more than was obtained from the 6-day control bottle that had never been sealed. The flasks and bottles that remained sealed failed to develop more than a trace of penicillic acid or of alcohol, even after two months, though more mycelium gradually developed.

This influence of aeration on the quality of the metabolism of Penicillium has not hitherto been noted. It furnishes a further explanation for some of the discordant results obtained with Penicillium. It shows, moreover, that the ferric-chlorid reaction when negative need not, as some have thought, give reliable information concerning the toxicity of a fungus. Under special conditions of oxygen supply, the reaction may become positive. Species of Penicillium which have not been found to give the reaction [2] will have to be reinvestigated under definite conditions of oxygen tension.

From media which are alkaline or become so, penicillic acid can not be isolated, perhaps because it is sensitive to alkali. Raulin's medium

[1] This method was very kindly suggested by Dr. Hideyo Noguchi, of the Rockefeller Institute of New York.

[2] Cf. supra, p.12.

is such when deprived of tartaric acid, or when it contains an equivalent amount of sodium acetate instead of tartaric acid, or when it contains 1 per cent of Witte's peptone as the source of nitrogen. Tartaric acid itself is unessential, since succinic acid may be substituted for it without changing the quantity of penicillic acid formed. The failure to find penicillic acid on peptone medium is not in harmony with the observation of Paladino-Blandini [1] that toxic phenolic substances are most abundantly formed on media containing nitrogen in the form of peptone.

The form in which nitrogen is offered may also be of influence, since when tyrosin or leucin were the only source of nitrogen no penicillic acid was detected. As these experiments were performed before the effect of the air supply was known, they are not conclusive. At any rate the absence of the acid from the tyrosin culture is remarkable, as penicillic acid is probably also an aromatic compound. The suggestion advanced by Audenino,[2] that some of the phenolic substances found in spoiled maize may be derived from the phenylalanin of the zein, does not receive support from these experiments on tyrosin.

On corn-meal mush the organism grew faster and fruited sooner than on Raulin's medium. Penicillic acid was formed, though the quantities extracted were decidedly smaller than those from Raulin's medium, perhaps because of the greater difficulties of purification. The alcoholic extract of the mush on evaporation left a red oil, like that reported by Lombroso.[3] From the water extract of the oil the acid crystallized on concentrating and cooling. These cultures gave but a weak or even negative ferric-chlorid reaction, showing that in detecting moldiness the unmodified reaction of Gosio is either not delicate enough or is inhibited by other substances present.

It is therefore evident that the amount of penicillic acid formed varies greatly with the conditions of growth. From 700 cubic centimeters of medium from the 2-liter, tightly plugged, Erlenmeyer flasks small quantities were separable at the end of the first week, about 0.1 gram at the end of the second, 0.2 gram the third, and 0.25 gram at the end of the fourth week, making the final concentration about 1 to 2,000. The method of separation probably does not isolate all the substance present.

TOXICITY OF PENICILLIC ACID.

Penicillic acid contains more oxygen than the material isolated by Gosio. This may explain why the former is toxic and the latter is

[1] Paladino-Blandini, A. Tossici di ifomiceti. Archivio di Farmacologia Sperimentale e Scienze Affini, v. 5, p. 606–664, 1906.

[2] Audenino, E. Etiologia della pellagra. Atti del Quarto Congresso Pellagrologico Italiano, Udine, 23–24–25 Septembre, 1909, p. 41. 1910.

[3] Lombroso, Cesare, and Erba, Carlo. Sulle sostanze stricniche e narcotiche del mais guasto. Reale Istituto Lombardo, Rendiconti, s. 2, v. 9, p. 133–147, 1876.

not, for it is a well-known fact that the introduction of hydroxyl into the benzol ring increases toxicity.[1] Benzol and phenol are good examples of this phenomenon.

The toxicity of penicillic acid is shown by the following experiments: When 7 milligrams of penicillic acid, dissolved in 0.5 cubic centimeter of physiological salt solution, are injected subcutaneously into a mouse of about 20 grams weight, convulsions and paralysis, particularly of the forelegs, develop very quickly, so that the head and forward portion of the body fall down on the ground while the hind quarters are still struggling and kicking. After a while these violent symptoms wear off and the animal is quiet; usually it assumes a sitting posture, with fur ruffled, the vessels of the ears congested, muzzle twitching, and diaphragm contracting spasmodically, so that the mouse has the appearance of retching. The animal might be regarded as recovering. However, death usually comes quietly in 5 to 12 hours. Apparently the symptoms are identical with those produced by the extracts of the corn cultures. A smaller dose (5 milligrams) causes the initial symptoms of convulsions and paralysis, but the animal usually recovers. Death is not due to acid action, for the same symptoms follow the injection of the same dose in solutions neutralized with sodium hydroxid. Moreover, the injection of equivalent quantities of hydrochloric or sulphuric acid is without serious effect. The fatal dose for mice is therefore about 0.3 to 0.35 gram per kilogram when injected subcutaneously.

With a stomach tube a guinea pig of 525 grams weight was fed 10 cubic centimeters of a 2 per cent solution of penicillic acid in warm normal saline solution. In the course of half an hour the animal became weak, particularly in the hind legs, and lay prone on the abdomen. When turned on the back he remained in that position. An hour later he began to recover. When returned to the cage he sat quietly without eating. The following day the weight had dropped to 495 grams, and on the second day to 470 grams. On the third day the weight rose to 485 grams and the animal recovered.

Another guinea pig of 410 grams weight received subcutaneously in the region of the spinal column 1.8 cubic centimeters of 2 per cent penicillic acid in warm normal saline solution. At first the effects were not marked. The animal remained quiet with fur bristling. Then a little weakness of the hind limbs developed. A second injection of 2 cubic centimeters was made 4½ hours later beneath the skin of the abdomen. In both injections an appreciable amount of the injected liquid was lost by oozing through the needle puncture. The animal was observed for 2½ hours after the second injection. No change in its condition was noted. The following morning it was found dead. Autopsy revealed nothing but rather extensive serous

[1] Fränkel, Sigmund. Die Arzneimittel-Synthese, Aufl. 2, Berlin, 1906, p. 53.

effusions at the sites of injection. There was neither pus nor other sign of severe inflammation. The right heart was dilated, the left ventricle strongly contracted. In two injections a third guinea pig, a female of 400 grams weight, received under the skin of the abdomen 0.1 gram of penicillic acid neutralized with sodium carbonate and dissolved in 1.5 cubic centimeters of normal saline solution. As soon as returned to the cage it began to feed, but an hour later was quiet and not very responsive to stimuli. It remained in this condition for an hour longer, when slight dyspnœa and signs of weakness appeared. An hour later the legs were weak, for the abdomen was allowed to rest on the ground. It still responded to stimuli, however, and did not remain on its back when turned over. Half an hour later its head was also resting on the ground. At the end of the fourth hour the weakness was so great it fell over on its side from time to time, but was able to right itself. Four and a half hours after the injection the animal sank over on its side and died without struggling. The autopsy showed that one of the injections had been made into the region of the functioning mammary gland. There was at this place a hemorrhagic effusion about an inch in diameter. There was a little serous exudation. The site of the other injection showed merely a little serous exudation. The animal was in an early state of pregnancy. Nothing else significant could be found.

Twelve milligrams of penicillic acid were dissolved in 1 cubic centimeter of normal saline solution by neutralizing with sodium carbonate. This was injected into the dorsal lymph sac of a frog of 45 grams weight. Though the frog was watched for 6 hours no symptoms were observed. Nevertheless, the following morning the animal was dead. The only thing noted on autopsy was that the heart was in systole.

A second frog of 35 grams weight received into the dorsal lymph sac 30 milligrams of penicillic acid dissolved with sodium carbonate. He soon began to get weak, without any other marked symptoms, and in 45 minutes had ceased breathing and was quite unresponsive. The body was at once opened. The auricles were still beating vigorously, but the ventricle was quiet in complete systole. It exhibited, only occasionally, slight fibrillation in the neighborhood of the auriculo-ventricular junction. This soon ceased, and 10 minutes later the auricles ceased pulsating.

Penicillic acid is also toxic to tadpoles. In a solution of 1 to 5,000 the animals seemed uncomfortable and restless as compared with the controls. There seemed to be considerable irritation, for the water became clouded with secreted mucus. At the end of 24 hours the animals were still in good condition and, when transferred to the aquarium, continued to live. In a solution of 1 to 2,000 similar

phenomena were observed, but after an hour there were occasional convulsive lashing movements. In the third hour the animals seemed to get weaker and the respiration became weak and shallow. At the end of 4 hours the animals no longer responded to stimuli, the respiration had almost ceased, and the animals did not recover when placed in the aquarium. In a solution of 1 to 1,000 the irritation and the lashing movements were more pronounced. For 2 hours there were fairly vigorous responses to stimuli; in 3 hours the animals were dead. The toxic effect was not observed when the tadpoles were placed in solutions of the neutral sodium salt. There was very little irritation, and they survived in solutions as strong as 1 to 1,000. For tadpoles the acidity of penicillic acid seems to be the chief factor in the toxic action, for in solutions of sulphuric and hydrochloric acid of a strength of 1 to 1,000 the animals were killed in 18 minutes. Moreover, in n/200 sulphuric acid, which has an acidity equivalent to that of a 1 to 1,000 solution of penicillic acid, the tadpoles died in an hour. These observations do not militate against the toxicity of penicillic acid, for it is probable that it is not to any considerable extent absorbed, because only minute traces, as has been shown, can be extracted from water by ether or by oil. The partition coefficient is too much in favor of water, and Overton [1] has shown that substances like these enter the organism very slowly. All these factors are still more effective in the case of solutions of the salt, which is quite insoluble in oils and most organic solvents. This is true in many other cases where the free acid is itself toxic aside from its acidity, but the salt quite harmless. Thus, Clark,[2] in whose paper the older literature is cited, states that the toxicity of acetic acid to Penicillium and certain other molds is due almost entirely to the un-ionized molecule CH_3COOH. Recently this fact has been rediscovered by Reichel.[3] It was found in this investigation that even in neutralized solutions of salicylic acid of 1 to 1,000 tadpoles are unaffected. Finally Gosio and Ferrati [4] state that neutralization of the toxic extracts prepared from their Penicillium cultures renders them much less toxic.

From all these data it appears that the toxicity of penicillic acid is of the same general magnitude as that of some of the phenols and phenol acids. According to Duplay and Maurice Cazin [5] the lethal dose of phenol for mice is 0.296 gram per kilogram. This is nearly as large

[1] Overton, E. Studien über die Narkose, zugleich ein Beitrag zur allgemeinen Pharmakologie, Jena, 1901, 195 p.

[2] Clark, J. F. On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Botanical Gazette, v. 28, p. 319, 1899.

[3] Reichel, J. Ueber das Verhalten von Penicillium gegenüber der Essigsäure und ihren Salzen. Biochemische Zeitschrift, Bd. 30, p. 152–159, 1911.

[4] Gosio, B., and Ferrati, E. Sull' azione fisiologica dei veleni del mais invaso da alcuni ifomiceti. Rivista d'Igiene e Sanità Pubblica, ann. 7, p. 961–981, 1896.

[5] Duplay, Simon, and Cazin, Maurice. De l'action de l'acide phénique sur les animaux. Comptes Rendus de l'Académie des Sciences [Paris], t. 112, p. 627–630, 1891.

as the lethal dose of penicillic acid. For guinea pigs and rabbits the lethal dose of phenol is about twice as much. For guinea pigs the lethal dose of penicillic acid seems to be smaller.

The data of Duplay and Cazin agree quite well with the findings of Paul Binet, who determined the lethal dose for rats at 0.5 to 0.6 gram and for guinea pigs at 0.55 gram per kilogram.[1] Gosio and Ferrati[2] state that 5 to 10 milligrams of phenol or resorcin and 15 milligrams of salicylic acid subcutaneously are fatal to mice.

Granting that penicillic acid is related to lichen acids, it becomes a matter of interest to note that certain lichen acids have been found toxic. Kobert[3] has found that vulpinic acid, a yellow substance found in *Evernia vulpina*, *Xantoria parietina*, and some other lichens, is a protoplasmic poison. He found both vulpinic and pinastric acid very toxic to frogs, and vulpinic and pinastric acids toxic to higher animals, while chrysophyscin is harmless. Zopf[4] found none of them except vulpinic acid injurious to orthoptera, arachnids, or helix. He does not think the lichen acids protect the lichen from attacks by animals, a view previously expressed by Bachmann[5] and Zukal.[6] Stahl,[7] however, believes that Zopf is only partly right.

Penicillic acid was found to resemble the crystalline substance of Gosio and the lichen acid, vulpinic acid, studied by Kobert,[8] in having some antiseptic power, as shown by its action on pure cultures of baker's yeast and of *Bacillus coli*. Yeast was chosen because it flourishes when the reaction is acid. *B. coli* was selected because it forms acid from sugar and is not harmed by considerable variations of the environment. The culture was kindly furnished by Dr. Rogers, of the Dairy Division of the Bureau of Animal Industry, being No. 22 f. g. of his collection. It had been isolated originally in the Hygienic Laboratory of the Public Health and Marine-Hospital Service.

Yeast.—Six flasks, provided with a trap containing sulphuric acid to prevent moisture losses, received each 25 cubic centimeters of sterile Raulin's medium. Three received in addition enough crystalline penicillic acid to make the concentration 1 to 1,000. All

[1] Binet, Paul. Toxicologie comparée des phénols. Revue Médicale de la Suisse Romande, ann. 15, p. 567, 631, 1895.

Prevost, and Binet, Paul. Travaux du Laboratoire de Thérap., Genf, 1896, p. 143. Cited in Fränkel, Sigmund, Die Arzneimittel-Synthese, Aufl. 2, Berlin, 1906, p. 55.

[2] Gosio, B., and Ferrati, E. Op. cit.

[3] Kobert, R. Über Giftstoffe der Flechten. Sitzungsberichte der Naturforscher-Gesellschaft. Dorpat, Bd. 10, p. 165, 1892.

[4] Zopf, W. Zur biologischen Bedeutung der Flechtensäuren. Biologisches Centralblatt, Bd. 16, p. 592–610, 1896.

[5] Bachmann, E. Ueber nicht-krystallisirte Flechtenstoffe, ein Beitrag zur Chemie und Anatomie der Flechten. Jahrbücher für Wissenschaftliche Botanik, Bd. 21, p. 17, 1890.

[6] Zukal, H. Morphologische und biologische Untersuchungen über die Flechten. Sitzungsberichte der Mathematisch-Naturwissenschaftliche Classe der Kaiserlichen Akademie der Wissenschaften [Wien], Abt. 1, Bd. 104, p. 529–574, 1303–1395, 3 pl., 1895.

[7] Stahl, Ernst. Die Schutzmittel der Flechten gegen Tierfrass. Festschrift zum Siebzigsten Geburtstage von Ernst Haeckel, Jena, 1904, p. 357. (Denkschriften der Medizinisch-Naturwissenschaftlichen Gesellschaft zu Jena, Bd. 11.)

[8] Kobert, R. Op. cit.

six were then inoculated with equal quantities of a suspension of the yeast and weighed. At intervals a pair of these flasks was heated to boiling and dry air drawn through them. They were cooled and weighed. The loss represents chiefly carbonic acid. The experiment was repeated, using a concentration of penicillic acid of 1 to 500. The results obtained are shown in Table III.

TABLE III.—*Loss in weight of flasks of Raulin's medium treated with various quantities of penicillic acid and inoculated with yeast.*

Time.	Loss in weight (grams) of flasks inoculated with yeast.			
	Proportion of penicillic acid 1 to 1,000.	Control (untreated).	Proportion of penicillic acid 1 to 500.	Control (untreated).
First day	0.4507	0.4537	0.3117	0.3566
Second day	.4820	.5482	.3535	.5308
Third day	.5293	.5720	.3550	.5755

In the control flasks no sugar was present after the third day. It was impossible to test the others because of the presence of penicillic acid, which is not destroyed by yeast but may be recovered almost quantitatively.

While these experiments do not disclose a powerful antifermentative action, still in both some decided effect is indisputable. While the yeast cells were not killed, there is an unmistakable retardation in the rate of fermentation. As far as could be judged by the naked eye, the multiplication of the cells was partly inhibited. At the end of the experiments there seemed to be much more yeast in the control flasks than in those containing penicillic acid. Whether the gross appearance corresponds to the facts will have to be determined by cell counts. It is not likely that these effects are due to the slight increase in acidity.

Bacillus coli.—For the culture medium 1 per cent acid bouillon was used. Series of test tubes were charged with 8 cubic centimeters. To the control tubes 1 cubic centimeter of normal salt solution was added. To the other tubes 1 cubic centimeter of a neutral solution of the sodium salt of penicillic acid was added of such a strength that the final concentration was 1 to 500. Both sets of tubes were sterilized for 20 minutes on three successive days in an Arnold sterilizer. A very dilute suspension of active bacteria was prepared and 1 cubic centimeter measured into each tube. The tubes were then incubated for 20 hours. When examined it was found that in the tubes containing penicillic acid there was very slight growth, whereas in the control tubes growth was abundant. The difference was so apparent that it was unnecessary to pour plates. As the neutral sodium salt

was used in this experiment there was no increased acidity, an objection which might be urged against the yeast experiments.

These experiments show that penicillic acid exerts some, though weak, antifermentative action. Stahl [1] has pointed out that the antiseptic power of vulpinic acid may be of considerable use to the lichen *Evernia vulpina* in protecting it from micro-organisms. One might advance a similar hypothesis for the secretion of penicillic acid. While it is likely that execretory products of one kind or another have this effect, their usefulness must be regarded as purely accidental and secondary, though it is not impossible that they play a part in selection. The theory that the formation of alcohol in the course of fermentation is for the purpose of suppressing competitors [2] must in this form be condemned.[3] One might as well maintain that the formation of carbonic acid is for the purpose of furnishing the yeast cell with a means of locomotion or for the purpose of creating the optimum acidity for the action of invertase.[4] It is true that alcohol and carbonic acid do these things for the yeast cell, but the phenomena are merely concomitant. The yeast experiments given above may indicate that penicillic acid has such a secondary effect, particularly when the oxygen supply to the organism is insufficient.

These observations on the antifermentative action of penicillic acid may furnish an explanation of the observation of Nikitinsky [5] that yeast will not grow on a medium on which Penicillium has been sown one or two days before, and vice versa. This is not due to the exhaustion of the medium, for replacing the sugar consumed does not alter the results. On the whole, Nikitinsky is inclined to regard the acids formed by the first organism as the agent inhibiting the growth of the second. He draws this conclusion because acids are the only inhibiting substances known to be formed. The discovery of penicillic acid renders it possible that some of these inhibitions are due to the formation of specific substances. They may be very generally formed, for Nikitinsky found the same inhibiting effect with other pairs of organisms than yeast and Penicillium.

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[1] Stahl, E. Op. cit., p. 374.

[2] Wortmann, Julius. Tätigkeit der Station in Bezug auf die Untersuchung und Behandlung kranker Weine. Bericht der Königl. Lehranstalt für Wein-, Obst-, und Gartenbau zu Geisenheim, p. 136, 1902.
Delbrück, M. Der physiologische Zustand der Zelle und seine Bedeutung für die Technologie der Gärungsgewerbe. Wochenschrift für Brauerei, Jahrg. 23, p. 513–516, 1906.
——— and Schönfeld, F. System der natürlichen Hefereinzucht, Berlin, 1903; p. 10.
Jost, Ludwig. Vorlesungen über Pflanzenphysiologie, Jena, 1904, p. 258.
Kienitz-Gerloff, Felix. Bakterien und Hefen, etc., Berlin, 1904, p. 35.

[3] Bierberg, W. Die biologisch-ökologische Theorie der Gärung. Centralblatt für Bakteriologie, [etc.], Abt. 2, Bd. 26, p. 187–189, 1910.
——— Alkohol- und Essigsäuretoleranz der Bakterien und die Wortmannsche biologische Gärungstheorie. Idem, Abt. 2, Bd. 24, p. 432–435, 1909.

[4] Hudson, Dr. C. S. Personal communication.

[5] Nikitinsky, J. Ueber die Beeinflussung der Entwickelung einiger Schimmelpilze durch ihre Stoffwechselprodukte. Jahrbücher für Wissenschaftliche Botanik, Bd. 40, p. 66, 1904.

It is not possible, however, to reach any definite opinion concerning his experiments, because they are complicated by the fact that he does not state how the medium was sterilized after the growth of the first organism. If sterilized with heat, it is possible that inhibiting substances were injured or volatilized.

A detailed study of the pharmacological action of penicillic acid on warm-blooded animals was not made, because to procure enough material would have unduly delayed publication. Such a study is reserved for a future paper. A single experiment indicating the effect on the blood pressure was performed, using a 6-kilogram dog in morphine-ether anæsthesia. The blood pressure in the carotid was recorded upon a kymograph in the usual way. In the course of 12 minutes 23 cubic centimeters of a 2 per cent solution of penicillic acid in physiological salt solution were introduced into the saphenous vein. Somewhat before the end of the injection the blood pressure began to fall and ultimately reached 65 per cent of the original value. Then it gradually recovered, until at the end of 10 minutes it had returned to its original value. During the stage of depression the heartbeat was slightly more rapid, but it also recovered.

It is a matter of considerable interest to compare the toxicity of penicillic acid with that of the extracts obtained by various investigators from various fungi. The only experiments strictly comparable with those on penicillic acid just recorded are those with Gosio's pure substance, in which no toxic effect was obtained. All other recorded experiments deal with impure mixtures. The extracts obtained by Gosio, Di Pietro, Sturli, and others were far more toxic than penicillic acid. This is particularly true of Di Pietro's "glucosid." One and a half milligrams of his impure extract caused within a quarter of an hour intense acute symptoms in a guinea pig of 300 grams.

Toxic preparations were obtained by Gosio's student, Paladino-Blandini,[1] from the toxic variety of *Penicillium glaucum* used by Gosio ("Var. ipertossico, Gosio"), from Oospora, Rhizopus, and Aspergillus. One does not, on carefully examining his protocols, get the impression that any of the organisms were very poisonous. This may have been due to the fact that his culture media were often alkaline or that he concentrated the media on the steam bath before testing for toxicity. If any of the organisms produced substances like penicillic acid these might have been destroyed either by the alkalinity or by the heating, for, as has been shown, penicillic acid is unstable under these conditions. Paladino-Blandini makes this point himself for the poison of *P. glaucum*, which he found to be ther-

[1] Paladino-Blandini, A. Tossici di ifomiceti. Archivio di Farmacologia Sperimentale e Scienze Affini, v. 5, p. 606-644, 1906.

molabile and volatile. Obviously in these experiments he was not dealing with *P. puberulum*.

Paladino-Blandini used these observations to explain some of the results of Ceni,[1] who claimed that the production of toxic or phenolic substances by various molds fluctuated with the season, being greatest in the spring and fall. Since Ceni always injected inspissated culture fluid or extracts, Paladino-Blandini suggested that the heating probably destroyed or volatilized the toxic substances, so that conditions were not constant. Di Pietro, too, was unable to confirm Ceni, for his organism was equally toxic all the year round. In the present investigation these results were checked by determining the amount of penicillic acid formed under like conditions at different times of the year. About the same yield of this acid was obtained in every month of the year. Against these observations must be set the statements of Otto [2] that extracts of Aspergillus cultures are toxic from April to October.

PHYSIOLOGICAL STUDIES OF PENICILLIUM PUBERULUM.

In order to obtain, if possible, better yields of penicillic acid, the organism was grown upon Raulin's medium, variously modified. The other conditions of growth remained the same as those previously used to procure penicillic acid.

The culture media employed may be classed in two groups: Those in which some component of Raulin's medium was replaced by an equivalent amount of another substance, and those in which some substance was added to Raulin's medium. The first group comprised the following substitutions:

Succinic acid for tartaric.

Sodium acetate for tartaric acid.

One and a half per cent ethyl alcohol for cane sugar and tartaric acid.

Leucin instead of nitric acid and ammonia. The amount of leucin was calculated to contain an equivalent amount of nitrogen. The leucin was pure and prepared by hydrolyzing plant protein, esterifying the resulting amino acids, subjecting them to fractional distillation, and saponifying.[3]

Tyrosin instead of nitric acid and ammonia. The tyrosin was prepared in the usual way by hydrolyzing horn with strong mineral acids.

One-tenth per cent amyl alcohol for cane sugar and tartaric acid.

Witte's peptone for nitric acid and ammonia.

The second group comprised the following:

Addition of 0.05 per cent amyl alcohol to Raulin's medium.

Addition of 0.01 per cent amyl alcohol to Raulin's medium.

Addition of 0.1 per cent amyl alcohol to Raulin's medium.

Most of the results have been collected in Table IV.

[1] Ceni, Carlo. Le proprietà tossiche di alcuni ifomiceti in rapporto colle stagioni e col ciclo annale dell' endemia pellagrosa. Comunicazione preliminare. Rivista Pellagrologica Italiana, v. 4, p. 89.

[2] Otto, M. Ueber die Giftwirkungen einiger Stämme von Aspergillus fumigatus und Penicillium glaucum nebst einigen Bemerkungen über Pellagra. Zeitschrift für Klinische Medizin, Bd. 59, p. 332–333, 1906.

[3] This preparation was very kindly furnished by Dr. I. K. Phelps, of the Bureau of Chemistry.

TABLE IV.—*Observations on the physiology of Penicillum puberulum.*

Medium.	Age of culture.[1]	Appearance of spores.[2]	Color of medium.	Weight of mycelium.	Volatile products.	Ethyl alcohol.	Acidity of 10 c. c. of medium.	Penicillic acid.	Remarks.
	Days.	*Day.*		*Grams.*					
Raulin's medium.	30–40	6th to 10th	Brown, dark, transparent.	8. Some already autolyzed.	No aldehyde; traces of acid, none with insoluble Ag. salts.	0.1 per cent	1.1 c. c. n/10 alkali.	About 0.2 gram in 700 c. c.	
Succinic acid	31	About the 6th	do	4.75	Traces of aldehyde and acetic acid.	Present	Not determined.	Present	
Sodium acetate.	31	10th traces, 15th abundant.	Darkening delayed.	Not weighed	Not studied	Not studied	Alkaline	Absent	Spore formation much delayed;[3] culture medium pale.
Ethyl alcohol 1.5 per cent.	70	About the 60th	Pale	Very slight	do	do	Not studied	Not tested	Spores did not germinate; after inoculation with mycelium very slow and scanty growth.
Witte's peptone.	25	9th	Very dark	Not weighed	Absent	Absent	Alkaline	Trace	A trace of an alkaloid, soluble in chloroform, present.
Leucin	33	8th	Light brown	7.7	No amyl alcohol; volatile acid-reducing Ag. present.	Present	0.6 c. c. n/10 alkali.	Absent	Very abundant spore formation.
Tyrosin	38	5th	do	4.25	No tyrol or tyrosol; volatile acid-reducing Ag. present.	Not studied	2.7 c. c. n/10 alkali.	do	Rapid, but very flat, thin, and delicate growth; strong musty odor.
Amyl alcohol:									
0.1 per cent									No growth.
0.05 per cent		6th to 10th	As in plain Raulin's medium.	Not weighed	Amyl alcohol present.	Present	Acid	Present	Growth good.
0.01 per cent		do	do	do	do	do	do	do	Do.
0.1 per cent									Almost no growth.

[1] The age refers to the interval between inoculation and chemical examination.
[2] The first tinge of green coloration visible to the naked eye was taken as a sign of spore formation. Many colorless or a few colored spores may have been formed sooner.
[3] Hasselbring (Botanical Gazette, v. 45, p. 170, 1908) reported abundant spore formation with potassium acetate as the only source of carbon.

A number of other facts were observed which have not been incorporated in the table. Thus it was found that in the cultures on Raulin's medium the tartaric acid soon disappeared. At least none could be isolated as the acid potassium salt. Indeed, the tartaric acid seems to be attacked at the beginning more rapidly than the sugar. Pfeffer [1] has reported analogous observations upon Aspergillus. Reichel [2] found very recently that Penicillium when grown in the presence of acetic acid begins to destroy the acid until the unfavorable acidity is reduced. A concentration of acetic acid equivalent to the tartaric acid of Raulin's medium was found to inhibit the development of *Penicillium puberulum*. That is why in one of the experiments of Table IV sodium acetate was used. There seems to be a general tendency for molds to reduce the acidity of a very acid medium. This may be done by destroying the acid, or, if the acid can not be attacked, by neutralizing it with ammonia when this can be formed by deamidization.[3] At any rate in the present instance the sugar disappeared more slowly than the tartaric acid. By the end of the fourth week, however, the medium was no longer optically active [4] or fermentable. Because penicillic acid renders the solution antiseptic the fermentation test is not in this instance reliable. Nevertheless the medium reduces Fehling's solution, 25 cubic centimeters yielding 78.1 milligrams of cuprous oxid.[5] The reduction is caused by penicillic acid.

Alcohol was determined by taking the specific gravity of the rectified distillate. The cultures contained 0.1 per cent as early as the end of the first week. The low concentration of alcohol found might not be the result of a scanty alcohol formation, but of the further oxidation of the alcohol formed, since alcohol is a good food for Penicillium.[6] Indeed, it has been said that "P. glaucum" does not produce alcohol at all.[7] Dox states that alcohol of a concentration not over 0.1 per cent is produced only when the air supply is insufficient.[8] All cultures of *Penicillium puberulum* tested contained alcohol, even those grown in the flat bottles above described, in which the aeration was certainly good.

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[1] Pfeffer, W. Ueber die Election organischer Nährstoffe. Jahrbücher für Wissenschaftliche Botanik, Bd. 28, p. 205–268, 1895.

[2] Reichel, J. Ueber das Verhalten von Penicillium gegenüber der Essigsäure und ihren Salzen. Biochemische Zeitschrift, Bd. 30, p. 152–159, 1911.

[3] Butkewitsch, Wl. Umwandlung der Eiweissstoffe durch die niederen Pilze im Zusammenhange mit einigen Bedingungen ihrer Entwickelung. Jahrbücher für Wissenschaftliche Botanik, Bd. 38, p. 198, 1902.

[4] The determinations were very kindly made by Dr. C. S. Hudson, of the Bureau of Chemistry.

[5] This determination was very kindly made by Dr. H. Hasselbring, of the Bureau of Plant Industry.

[6] Hasselbring, Heinrich. The carbon assimilation of Penicillium. Botanical Gazette, v. 45, p. 170–193, 1908.

[7] Brefeld, Oscar. Ueber Gährung. III. Vorkommen und Verbreitung der Alkoholgährung im Pflanzenreiche. Landwirthschaftliche Jahrbücher, Bd. 5, p. 315, 1876.

[8] Dox, A. W. The intracellular enzyms of Penicillium and Aspergillus, with special reference to those of Penicillium camemberti. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 120, p. 33. 1910.

No glycerin could be detected in the culture fluid when 200 cubic centimeters, rendered weakly alkaline with sodium carbonate, were concentrated to a sirup, the sirup extracted with alcohol, and the alcoholic extract after evaporation tested with the borax bead.

Though the Raulin medium cultures remained distinctly acid to litmus, no nonvolatile acid other than penicillic acid could be extracted with sulphuric ether, petroleum ether, or acetic ether, even after acidifying with phosphoric acid. No insoluble lead, copper, calcium, barium, or zinc salt could be obtained. Great care was taken to detect oxalic acid, but in young cultures none could be found. Even in old cultures none could be detected by the ordinary method of extraction with ether. A small amount was isolated in the following manner: Seven hundred cubic centimeters of culture medium about two months old were concentrated to a sirup, acidified with phosphoric acid, and mixed with clean sand and plaster of Paris. When the plaster had set, the mass was ground and extracted with ether in a Soxhlet extractor. Oxalic acid, identified by its melting point, crystallized from the extract, which also contained other material, as shown by the evolution of gas and the odor of nitrous oxid. Apparently some nitric acid passed from the medium into the extract and there caused decomposition. The extract contained a substance soluble in chloroform and giving a bright-green color with ferric chlorid. An alkaline solution of penicillic acid when concentrated to a sirup does not yield oxalic acid, but it does give a green color with ferric chlorid. Fumaric acid was absent. As already indicated, the culture medium contained unidentified substances. This was further shown by the fact that by the method of Griess, using sodium nitrite, sulphanilic acid, and acetic acid, an azo dye of a beautiful carmine color was produced. This reaction was obtained by Raciborski[1] with a number of fungi.

Only very small quantities of volatile material other than alcohol were detected in the culture medium. For the purposes of this examination a culture medium from which the penicillic acid had been removed as thoroughly as possible with chloroform to avoid obtaining its decomposition products, was used. The medium was then distilled and the distillate extracted, first with chloroform and then with ether. The residue from the chloroform consisted of a few small white crystals with a melting point of 112° C. The residue from the ether consisted of a few fine hairlike crystals. None of the crystals gave the ferric-chlorid reaction. Distillation of this liquid with mineral acid yielded no other products. Distillation with

[1] Raciborski, M. Über die Assimilation der Stickstoffverbindungen durch Pilze. Bulletin International de l'Académie des Sciences de Cracovie. Classe des Sciences Mathématiques et Naturelles, ann. 1906, p. 733–770. 1907.

alkali yielded a few white crystals obtained by extracting the distillate with ether.

All these experiments refer to the unmodified Raulin's medium. The mycelium was also studied. From mycelium of varying age grown under different conditions no toxic material could be extracted with boiling alcohol. An oily, waxy residue remained after the alcohol was removed on the steam bath. When this residue was extracted with water and the extract injected subcutaneously into mice no serious toxic effects were observed.

Mannitol has long been known as a constituent of Penicillium.[1] Trehalose and trehalum, or substances resembling them, have also been described. It is possible that in fungi some investigators may have mistaken trehalum for starch or glycogen.[2] Cramer [3] found that by treating the spores of Penicillium with boiling water no carbohydrate material precipitable by alcohol passed into the extract. When, however, the spores were exhausted with ether before the extraction, a carbohydrate was obtained giving a deep blue color with iodin. This carbohydrate, Cramer thought, resembled hemicellulose, but it may have been similar to trehalum. Moreover, a direct relationship between mannitol, trehalose, and trehalum has been demonstrated, trehalose being formed only at certain stages of growth, while later only mannitol occurs.[4]

Mannitol was readily detected in the mycelium of *Penicillium puberulum* by extracting the mycelium dried in air with boiling alcohol. On cooling, sweet, fine, white, silky needles separated, soluble in water and alcohol, insoluble in chloroform, and with a melting point of 162° to 163° C., uncorrected. Cholesterol reactions were negative. They did not reduce Fehling's solution, though they did so after oxidation with nitric acid. They rotated polarized light slightly to the left.

In the mycelium dried in air neither trehalose nor trehalum could be detected. When fresh mycelium was immersed in boiling alcohol, as soon as removed from the culture flask and the boiling extract filtered, no trehalose separated on cooling. This extracted mycelium, boiled with water and filtered hot, gave, on cooling, a small quantity of gummy material which iodin colored intensely violet and which was not easily inverted by hot dilute hydrochloric acid. This substance is plainly trehalum, in no way mistakable for glycogen or the more readily soluble and easily inverted starch.

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[1] Zopf, Wilhelm. Die Pilze, Breslau, 1890, p. 125.

[2] Lippmann, E. O. von. Die Chemie der Zuckerarten, Aufl. 3, Halbbd. 2, Braunschweig, 1904, p. 1429–1430.

[3] Cramer, E. Die Zusammensetzung der Sporen von Penicillium glaucum und ihre Beziehung zu der Widerstandsfähigkeit derselben gegen äussere Einflüsse. Archiv für Hygiene, Bd. 20, p. 197–210, 1894.

[4] Lippmann, E. O. von. Op. cit., p. 1427.

Both the culture fluid and the mycelium were examined for oxidizing enzyms. The former contains an abundance of catalase, though no oxidase detectable by guajac, aloïn, or benzidin. A very faint peroxidase reaction was found, due perhaps to the presence of chlorids.[1] The statement of Loew [2] that filtered *Penicillium glaucum* cultures contain only catalase is therefore amply confirmed. Fresh and air-dry mycelia were ground in a mortar with distilled water and allowed to digest at room temperature for several hours. The extract, filtered through paper, contained far more catalase than the culture medium, but neither oxidase nor peroxidase could be detected by the color tests. In performing these tests great care was taken to vary the reaction, for this has been shown to influence these tests greatly.[3]

The mycelium was then tested for oxidizing power by the method of oxygen absorption as developed in this laboratory by Dr. H. H. Bunzel.[4] The air-dry mycelium was ground and the dry powder obtained used directly in the oxidase apparatus in the presence of pyrogallol and of tyrosin. No oxygen absorption was observed. To make certain that neither the drying nor the acid of the medium was accountable for the negative results, the organism was grown on a medium containing monosodium phosphate and disodium phosphate, which, as shown by Henderson and Webster,[5] remains neutral. This medium was Raulin's solution, to which was added 5 per cent of a mixture of two parts Na_2HPO_4 and one part NaH_2PO_4. On this medium few spores developed in 10 days. The mycelium remained colorless. The medium contained alcohol and only traces of penicillic acid. Ten grams of perfectly fresh mycelium of 12 days' growth were ground with clean sand and then transferred to the absorption flask, together with 4 cubic centimeters of 10 per cent pyrogallol. This concentration of pyrogallol was selected because of its antiseptic action. Under these conditions no oxygen absorption was observed for more than an hour. Absorption then gradually began. This phenomenon is still under investigation.

Some of the results obtained with the variously modified Raulin's medium require further comment.

In the distillate from the succinic-acid culture aldehyde was detected by the power to reduce ammoniacal silver, and acetic acid by the formation of the ethyl ester with its characteristic odor.

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[1] Alsberg, C. L. Beiträge zur Kenntnis der Guajak-Reaktion. Archiv für Experimentelle Pathologie und Pharmakologie, Festschrift Schmiedeberg, Supplementband, p. 39, 1908.

[2] Loew, Oscar. Catalase, a new enzym of general occurrence, with special reference to the tobacco plant. U. S. Department of Agriculture, Report 68, 1901.

[3] Alsberg, C. L. Op. cit.

[4] Bunzel, H. H. The measurement of the oxidase content of plant juices. U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 238, 1912.

[5] Henderson, L. J., and Webster, H. B. The preservation of neutrality in culture media with the aid of phosphates. Journal of Medical Research, v. 16, p. 1-5, 1907.

The observation made on the 1.5 per cent ethyl-alcohol culture that mycelium failed to develop from spores, whereas inoculation with pieces of mycelium resulted in growth, is in harmony with the statement of Duclaux [1] that while alcohol restrains or arrests the germination of mold spores it is utilized almost as abundantly as sugar by the adult plant. These observations were confirmed by Clark.[2] The mycelium of *Penicillium puberulum* developed very slowly from mycelium inoculation on 1.5 per cent alcohol. In the course of a few weeks a delicate, thin, green growth spread over the surface of the medium. Except for the green color it had the appearance of the scum of lead oxid that forms on the surface of molten lead exposed to the air.

The peptone cultures turned exceedingly dark. When the alkaline medium was extracted with chloroform, the residue of the extract consisted of a little oil which was for the greater part soluble in acid. The acid solution gave a decided precipitate with Meyer's reagent for alkaloids. The small quantity available was not toxic to mice. When the medium was acidified before extraction a little nontoxic oil passed into the chloroform. By extracting with warm water no penicillic acid was obtained. However, on long standing with ferric chlorid the extract developed a faint rose color.

The purpose of the leucin and tyrosin cultures was to ascertain whether *Penicillium puberulum* is able to deamidize amino acids as yeast does. If this were the case amyl alcohol would have been found in leucin cultures and tyrosol or tyrol in tyrosin cultures.[3] Amyl alcohol was sought by the method of Beckmann,[4] and tyrol and tyrosol by the method of Ehrlich.[5] Raciborski [6] first observed differences in the manner in which different molds decompose tyrosin. He found that *P. glaucum* grown on tyrosin agar produced a substance reducing silver. Since both leucin and tyrosin are destroyed by *P. puberulum*, it is evident that either their decomposition differs from that to which these amino acids are subjected by yeast, or else amyl alcohol, tyrol, and tyrosol are merely intermediary products.

[1] Duclaux, E. Sur la nutrition intracellulaire. Annales de l'Institut Pasteur, ann. 3, p. 97–112, 1889.

[2] Clark, J. F. On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Botanical Gazette, v. 28, p. 385, 1899.

[3] After the completion of these experiments, Ehrlich and Jacobsen reported that *Penicillium glaucum* is able to decompose amino acids to simpler compounds of lower molecular weights. Still more recently, Herzog and Saladin published similar results. See Ehrlich, Felix, and Jacobsen, K. A., Über die Umwandlung von Aminosäuren in Oxysäuren durch Schimmelpilze. Berichte der Deutschen Chemischen Gesellschaft, Jahrg. 44, p. 888–897, 1911; Herzog, R. O., and Saladin, O., Über das Verhalten einiger Pilze gegen Aminosäuren, Zeitschrift für Physiologische Chemie, Bd. 73, p. 302–307, 1911.

[4] Beckmann, Ernst. Zur Bestimmung des Fuselölgehaltes alkoholischer Flüssigkeiten. Zeitschrift für Untersuchung der Nahrungs- und Genussmittel, Bd. 10, p. 143–152, 1905.

[5] Ehrlich, Felix. Über die Vergährung des Tyrosins zu *p*-oxyphenyl-äthylalkohol (Tyrosol). Berichte der Deutschen Chemischen Gesellschaft, Jahrg. 44, p. 139–146, 1911.

[6] Raciborski, M. Über die Assimilation der Stickstoffverbindungen durch Pilze. Bulletin International de l'Académie des Sciences de Cracovie. Classe des Sciences Mathématiques et Naturelles, ann. 1906, p. 767. 1907.

That the latter is probably the case is indicated by the fact that penicillic acid is formed abundantly only under conditions of imperfect aeration. Perhaps under these conditions tyrosin and leucin would be less completely oxidized. It certainly is significant that yeast which grows anaerobically produces tyrosol or *p*-oxyphenyl alcohol from tyrosin, a substance which resembles penicillic acid in bitter, toxic,[1] and certain chemical properties. Another observation of Raciborski[2] suggests a different explanation of the results. He found that *Aspergillus niger* in a condition of carbon hunger decomposes tyrosin in a different way than when it is plentifully supplied with sugar. It is possible that since *P. puberulum* was allowed to grow more than a month it was, during the latter period of its growth, in a state of carbon starvation because all the sugar had been consumed.

The amyl-alcohol cultures were designed to learn whether this alcohol could be oxidized by *P. puberulum.* It was thought that in this way some indication might be given showing whether it was an intermediary product in the decomposition of leucin. Unfortunately, as inspection of Table IV shows, amyl alcohol is so poisonous that the question can not be definitely settled in this way. The percentage tolerated is so small that the quantity can not be determined with sufficient accuracy. It may, however, be stated that there is no evidence that amyl alcohol is oxidized by *P. puberulum,* since after several weeks' growth it had not disappeared from cultures containing as little as 0.05 per cent. In these cultures it was separated by the method of Beckmann, the characteristic odor being recognized in the final extract. These observations probably have little bearing on the question of the intermediary formation of amyl alcohol from leucin. As far as they indicate anything they are not in favor of it.

In both leucin and tyrosin cultures an appreciable amount of volatile acid was found, possibly formic acid, since the silver salt was rapidly reduced. Because of this property the acid from the leucin culture was not studied. That from the tyrosin culture formed a crystalline barium salt. The quantity was too small for analysis.

The tyrosin culture presented a number of peculiarities. While the musty odor of the growth on ordinary Raulin's medium was perceptible only when the mycelium was held close to the nose, that on tyrosin had a typical and extremely musty odor which permeated the room when the flask was opened. The odor of ordinary cultures became more perceptible when they were distilled, since the odoriferous

[1] Ehrlich, Felix. Über die chemischen Vorgänge des pflanzlichen Eiweissstoffwechsels und ihre Bedeutung für die alkoholische Gärung und andere pflanzenphysiologische Prozesse. Landwirthschaftliche Jahrbücher, Bd. 38, Ergänzungsbd. 5, p. 306–307, 1909.

[2] Raciborski, M. Op. cit., ann. 1906, p. 765. 1907.

principle seemed to accumulate in the distillate. Thom [1] observed that the production of odors varied greatly not merely from species to species, but also in some individual species according to the culture medium. It would be interesting to investigate whether in the present case the production of the odorous principle was actually dependent upon the presence of an aromatic compound or upon some other condition. Such factors as these may be involved in the production of the flavor of cheese.

The rate of growth was very much more rapid at first than in the controls, in spite of the fact that little more than half the tyrosin was consumed. Moreover, spore formation began before the fifth day and was very abundant. In less than a week the entire surface of the liquid was covered with mycelium uniformly green with spores. It was more delicate and less stiff and woody than that of the controls. It was smooth and even, and lay flat on the surface, whereas in the controls it was convoluted and twisted so that some of it was pushed below the surface, resulting in the formation of new mycelium above. In the tyrosin the growth, after the surface had once been covered, seemed less bulky, although the old mycelium was gradually overgrown with new mycelium.

Certain of the facts here recorded have some theoretical interest. This is particularly true of the peculiarities of growth of the tyrosin culture and of the absence of easily detectable oxidizing enzyms in all the cultures tested.

The peculiarities of the tyrosin culture which are of interest in this connection are the thinness of the mycelium and precocity of the spore formation. Tyrosin contains the aromatic ring. Perhaps a large amount of aromatic derivatives is required so that spore formation can not take place until the organism has had time to manufacture these aromatic compounds from the sugar and other straight-chain carbon compounds offered. When an assimilable aromatic compound is offered, this latent period is perhaps bridged over. Certainly, on the tyrosin the spore formation is at least as rapid as on corn-meal mush, which, in its proteins, contains an abundance of aromatic compounds. The importance of various amino acids for microbic growth has recently been brought out in studies on the cultivation of the leprosy bacillus.[2] Another explanation may, however, be based on the assumption that spore formation is rapid in an exhausted or unfavorable medium. That this is actually true is still an open question, though Tiraboschi [3] presents

[1] Thom, Charles. Cultural studies of species of Penicillium. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 118, p. 90, 1910.

[2] Duval, C. W. Cultivation of the leprosy bacillus from the human tissues, with special reference to the amino acids as culture media. Journal of Experimental Medicine, v. 13, p. 365-373, 1911.

[3] Tiraboschi, C. Studi sugli ifomiceti parassiti del granturco guasto. Atti del Terzo Congresso Pellagrologico Italiano, Milano, 24-25-26 Septembre, 1906, p. 138. 1907.

evidence for this view. If this hypothesis be accepted it would be necessary to assume that tyrosin is an unfavorable source of nitrogen, either because of its chemical properties or because of its insolubility. That its chemical properties are the cause seems hardly likely, since leucin, which also contains amino nitrogen, gives such an abundant growth, although Raciborski [1] states that tyrosin is a much poorer source of nitrogen for *Aspergillus niger* than ammonia. That its insolubility is the cause is quite possible. Tyrosin is so little soluble that the amount in solution at any moment would be very much less than in the unmodified Raulin's medium, for most of the tyrosin contained in the culture had crystallized out on the bottom of the flask, at least 5 centimeters below the mycelium. Hence, it is possible that the rapid sporification was a sign of nitrogen starvation, which is the more likely, as the culture was very little handled or agitated. Under these conditions diffusion is very slow indeed, particularly for substances of great molecular weight like tyrosin. That diffusion does not keep pace with consumption of material is evident if any flask culture which has remained undisturbed for some days be examined. If such a culture be held between the eye and the light and gently rotated, convection currents can be seen near the mycelium, showing that the specific gravity of the liquid in immediate contact with the mycelium is very much less than that of the deeper layer. It may therefore be that after the first few days the mycelium was in relative nitrogen starvation. This hypothesis would account for the rapid initial and slow subsequent growth.

A perusal of the literature on the metabolism of the fungi shows that this question of the rate of diffusion has not received adequate attention. If the organism is grown on a thick layer of culture fluid the rate of diffusion of the dissolved substances is undoubtedly an important factor. A substance of small molecular weight will diffuse upward more rapidly to replace that consumed than one of great molecular weight. Thus the substance of small molecular weight might appear to be a better food relatively than it really is. The same logic applies to metabolic products. When these products are substances like alcohol, of light specific gravity, they will accumulate at the surface, particularly if the viscosity of the culture fluid is great, owing to the presence of much sugar or peptone. If, then, the alcohol concentration be determined for the whole fluid the values obtained may be spurious. Actually the mycelium may have been in contact with a much higher concentration of alcohol. This may be one of the reasons why the crop of mycelium for a given amount of sugar is said to be greater when the organism is grown on a thin

[1] Raciborski, M. Über die Assimilation der Stickstoffverbindungen durch Pilze. Bulletin International de l'Académie des Sciences de Cracovie. Classe des Sciences Mathématiques et Naturelles, ann. 1906, p. 764. 1907.

layer with great surface than when grown on a thick layer of culture fluid with smaller surface.[1] The largest yields were obtained in this way.

The absence of easily detectable oxidase is significant in connection with the discovery of Euler and Bolin that the oxidase of alfalfa (*Medicago sativa*) is a mixture of the calcium salts of simple oxyacids.[2] It is believed by some that many molds do not require calcium.[3] It is tempting to imagine a connection between the absence of easily detectable oxidase, the observation of Euler and Bolin, and the absence of calcium.

GENERAL CONSIDERATIONS.

Since it has been definitely shown in the present paper that a distinct species of Penicillium produces a substance of moderate toxicity, the question very naturally arises, has it any pathological significance? At present it can only be said that it is too early to answer this question. All that can be done is to discuss the possibility and to indicate further work to be done.

In acute intoxications, alleged to be due to molds, penicillic acid alone can hardly be of significance. The lethal subcutaneous dose for mice, as has been shown, is about 0.3 gram per kilogram of body weight. Assuming the same susceptibility for the average man of about 70 kilos, the dangerous dose would probably be about 21 grams. Hence, an acute intoxication from penicillic acid would require that an inconceivably great quantity of moldy food be consumed in a single day. Even herbivorous animals could hardly eat enough moldy fodder in a day to be acutely affected by penicillic acid. It is quite out of the question that in the natural course of events penicillic acid is likely to produce acute intoxication.

However, the results of this investigation of *Penicillium puberulum* indicate the possibility of acute intoxication by moldy food. As already stated, the different species of Penicillium differ radically in their biochemical behavior. If there is so much difference in the ordinary products of metabolism, it is altogether likely that a series of toxic substances may be produced by different species. Some of these substances might very well be far more toxic than penicillic acid. Indeed, Italian investigators have shown this contingency to be very probable. Gosio, Di Pietro, and Sturli have obtained from pure cultures of Penicillium toxic extracts far more poisonous than

[1] Nikitinsky, J. Ueber die Beeinflussung der Entwickelung einiger Schimmelpilze durch ihre Stoffwechselprodukte. Jahrbücher für Wissenschaftliche Botanik, Bd. 40, p. 43, 1904.
Raciborski, M. Op. cit., p. 733–734.

[2] Euler, H., and Bolin, I. Über die chemische Zusammensetzung und die biologische Rolle einer Oxydase. Zeitschrift für Physikalische Chemie, Bd. 69, p. 187–202, 1909.

[3] Loew, Oscar. Über die Giftwirkung von oxalsauren Salzen und die physiologische Funktion des Calciums. Biochemische Zeitschrift, Bd. 38, p. 226–243, 1912.
Robert, Mlle. Influence du calcium sur le développement et la composition minérale de l'Aspergillus niger. Comptes Rendus de l'Académie des Sciences [Paris], t. 153, p. 1175–1177, 1911.

anything hitherto obtained in the new work herein recorded. Because none of these investigators have isolated the toxic principle in a state of purity their researches have not been given the serious consideration that is their due. It will be the task of this laboratory to extend these investigations to other species of Penicillium in the hope that other toxic substances, perhaps more active than penicillic acid, may be isolated.

In the matter of chronic intoxication the situation is quite different. Continued use of moldy food containing penicillic acid might produce symptoms. The quantity of badly spoiled corn-meal mush which a man would consume at a single meal might contain as much as 0.1 to 0.5 gram of penicillic acid. As this acid has a toxicity of the same order of magnitude as phenol, resorcin, or salicylic acid, and as such substances are believed by many to be undesirable as food preservatives, it seems reasonable to demand that great care be exercised in eliminating moldy corn from the diet. Owing to the difficulty of procuring material, it has not been possible to conduct long-continued feeding experiments. Therefore it is impossible to say whether penicillic acid has cumulative action. Should it prove to have such action chronic intoxication might be brought about by comparatively small doses. For this reason it is very desirable to learn the constitution of penicillic acid in order to be able to make it synthetically. This is the most promising way to obtain larger quantities of it.

While the finding of penicillic acid indicates that the relation of moldy corn to pellagra[1] deserves renewed attention, this discovery does not materially strengthen the maize theory of the etiology of pellagra. Penicillic acid itself is not sufficiently toxic. It is quite possible that penicillic acid or a closely related substance may have been responsible for the toxic effects following the administration of "pellagrozein," the poison obtained from spoiled maize, with which, according to the experiments of Lombroso,[2] the disease could be produced artificially. "Pellagrozein" itself Lombroso did not regard as anything but a mixture. He believed it contained two alkaloids, which accounted for the toxic action. Neither alkaloid has ever been obtained in a state of purity, so that it is impossible to form any definite opinion about them. Indeed, other investigators have not been able to find alkaloids at all in spoiled maize.[3] It is quite

[1] Cf. Marie, A., Pellagra, authorized translation from the French, by C. H. Lavinder and J. W. Babcock, Columbia, S. C., 1910.

[2] Lombroso, Cesare, and Erba, Carlo. Sulle sostanze stricniche e narcotiche del mais guasto. Reale Istituto Lombardo, Rendiconti, s. 2, v. 9, p. 133–147, 1876.

[3] Monselise, G. Ricerche chimico-tossicologiche intorno ad alcuni campioni di mais per la studio della pellagra, Mondovi, 1881, 58 p. (Cited by Gosio.)

Selmi, Antonio. Delle alterazioni alle quali soggiace il granturco (Zea mais) e specialmente di quello che ingenera la pellagra. Atti della R. Accademia dei Lincei, s. 3, Memorie della Classe di Scienze Fisiche, Matematiche e Naturali, v. 1, dispensa 2, p. 1099–1141, 1877.

Di Pietro, Melchiorre. Sui veleni di alcune muffe. Annali d'Igiene Sperimentale, v. 12 (n. s.), p. 314–365, 1902.

possible that these alkaloids were either normal constituents of maize or ptomainelike bases, as was pointed out by Pelloggio,[1] for the investigators who found alkaloids seem to have often allowed the maize to spoil to an extreme degree. In one case the maize was actually allowed to rot until it stank.[2] If the maize used contained either penicillic acid or some similar substance the method of preparation was such that these substances would have passed into the "pellagrozein," which is even less toxic than penicillic acid. The lethal subcutaneous dose varied from 1.5 grams per kilogram for frogs to 7 to 10 grams for cats.[3] Hence, it is not impossible that "pellagrozein" contains substances of this type. Whatever evidence there is for the relation between "pellagrozein" and pellagra would apply equally well to penicillic acid. The discussion of this question lies beyond the scope of the present paper.

PENICILLIUM STOLONIFERUM.

In a former publication [4] it was stated that most of the samples of spoiled American maize examined in this laboratory failed to give Gosio's [5] phenol test with ferric chlorid. In this respect American spoiled maize seems to differ from that found in Italy, where the ferric-chlorid reaction is regarded as a reliable test for the deterioration of maize.[6]

Since the publication of the above-cited studies of the deterioration of maize the test has been improved in this laboratory so that in its new modification it is more delicate. The procedure as now conducted consists in extracting 50 grams of ground corn or meal in a stoppered flask, with sufficient chloroform to cover the mass. After two hours the chloroform extract is filtered off and concentrated to a bulk of 10 to 15 cubic centimeters. This concentrate is transferred to a small separatory funnel or test tube and covered with 5 cubic centimeters of water containing a trace of ferric chlorid. If substances like penicillic acid are present, the characteristic color develops in the aqueous layer.

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[1] Pelloggio, Pietro. Materia reagente quale alcaloide, trovata nell' estratto del mais guasto preparato dall' Erba. Reale Istituto Lombardo, Rendiconti, s. 2, v. 9, p. 118–121, 1876.

[2] Lombroso, Cesare, and Erba, Carlo. Loc. cit.

Biffi, S. Sulla nota del prof. Cesare Lombroso: I veleni del mais e la pellagra. Reale Istituto Lombardo di Scienze e Lettere, Rendiconti, s. 2, v. 9, p. 282–288, 1876.

[3] Lombroso Cesare. I veleni del mais e la loro applicazione all'igiene ed alla terapia. Rivista Clinica di Bologna, s. 2, ann. 7, p. 109–112, 1877.

—— and Erba, Carlo. Op. cit.

[4] Black, O. F., and Alsberg, C. L. The determination of the deterioration of maize, with incidental reference to pellagra. U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 199, 1910.

[5] Gosio, B. Ricerche batteriologiche e chimiche sulle alterazioni del mais. Rivista d' Igiene e Sanità Pubblica, ann. 7, p. 825–849, 869–888, 1896.

[6] Gosio, B. Alterazioni del granturco e loro profilassi. Italy, Direzione generale dell' Agricoltura, Annali di Agricoltura, no. 261, 1909.

When the tests are conducted in this way the number of samples of obviously deteriorated maize showing the reaction is greater than when the unmodified Gosio test is employed. Nevertheless, a positive result seems to be less frequent in American maize than in Italian maize. Moreover, the colors obtained with American spoiled maize have always been found to be red or red brown, while in Italy tests of spoiled corn are most commonly described as showing violet, blue, purple, and greenish tints. None of these tints have been encountered in American maize in this laboratory.

Since this sharp difference apparently exists between American and Italian deteriorated maize, it is desirable to compare samples of Italian spoiled maize with American ones. Opportunity to make this comparison was offered by Dr. C. H. Lavinder, of the Public Health Service, who while on a visit to Italy very kindly secured samples of condemned maize.

From one of these samples of maize Dr. E. F. Smith, of this Bureau, isolated two species of Penicillium. One of these species was identified by Dr. Charles Thom, of the Storrs Agricultural Experiment Station, as *P. stoloniferum* Thom.[1]

This organism when grown on Raulin's medium gives the very strong and characteristically violet ferric-chlorid reaction of Gosio. It is certainly a remarkable fact that the first sample of spoiled Italian corn examined gave the violet color described by Italian authors, whereas no American sample has been found giving a similar tint.

It was therefore decided to isolate, if possible, the substance responsible for the ferric-chlorid reaction. For this purpose the organism from Italian spoiled corn was grown in "Long Blake" bottles on Czapek's medium and on Raulin's medium in the manner above described. It was found that the organism grew more rapidly upon Raulin's medium. Therefore, for the preparation of material Raulin's medium only was used.

The substance responsible for the ferric-chlorid reaction was isolated by the following procedure: The culture fluid and the mycelium were transferred to an evaporating dish and rendered weakly alkaline with sodium carbonate. The contents of the dish were then heated to boiling and filtered hot. The mycelium remaining on the filter was thoroughly expressed. The mass was then again extracted with water rendered weakly alkaline with sodium carbonate. The combined extracts were evaporated to a small bulk over a free flame and filtered hot. To the clear filtrate a slight excess of hydrochloric acid was added. An abundant precipitate

[1] Thom, Charles. Cultural studies of species of Penicillium. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 118, 1910.

was produced, which consisted of a mixture of needle clusters and amorphous material. The precipitate was separated by filtration and washed with cold water. After drying spontaneously it was extracted with hot toluene and the hot extract filtered. Only the crystalline portion of the precipitate dissolved. The amorphous dark-brown material which remained on the filter was discarded, for it did not give a color reaction with ferric chlorid. On cooling and evaporating, the toluene extract spontaneously precipitated in the form of needles, the material giving the ferric-chlorid reaction. These needles, which were still slightly colored, were finally obtained white either by decolorizing with boneblack in hot toluene solution or by dissolving in alcohol and adding alcoholic potassium hydroxid to form the potassium salt, which is insoluble in alcohol. This salt was then washed free from color with alcohol. From the potassium salt the free acid was recovered in the form of white needles by dissolving the salt in water and precipitating with hydrochloric acid.

The substance thus obtained consists of white needles with a melting point of 140° C., uncorrected. The name mycophenolic acid is provisionally suggested for it. It is almost insoluble in water, but freely soluble in alcohol, in ether, and in chloroform. It is somewhat less soluble in benzene, only moderately soluble in cold toluene, and very soluble in hot toluene. With ferric chlorid it gives a violet color in aqueous solution, though its solubility in water is not sufficient to render the color intense. In alcoholic solution it gives a bright-green color with ferric chlorid. It does not react with Millon's reagent. It does not give Lieberman's reaction and could not be diazotized. It does not reduce Fehling's solution nor ammoniacal silver nitrate. It is fairly resistant to sodium, ammonium, and potassium hydrates and to hydrochloric, sulphuric, and acetic acids, being unaffected by boiling in 10 per cent solutions of any of these reagents. It does not contain water of crystallization. Its salts of potassium and sodium are very soluble in water. The salt of potassium is soluble in dilute alcohol, but insoluble in absolute alcohol. The salt of sodium is soluble in absolute alcohol, but may be precipitated in crystalline form by adding ether. The salt of barium is only very slightly soluble in water and forms clusters of minute needles. The copper, lead, and silver salts are amorphous and insoluble in water. In characterization of the substance the facts collected in Table V were ascertained by analysis of the free acid, by titration of the alcoholic solution of the free acid with n/10 sodium hydroxid, and by the determination of the barium content of the salt on ignition in platinum with sulphuric acid.

TABLE V.—*Analyses of mycophenolic acid.*

Weight of substance.	CO_2.	H_2O.	C.	H.	$BaSO_4$	Ba.	N/10NaOH.
Gram.	*Gram.*	*Gram.*	*Per cent.*	*Per cent.*	*Gram.*	*Per cent.*	*C. c.*
0.2316	0.5419	0.1315	63.81	6.30			
.2044	.4770	.1161	63.64	6.31			
.2494					0.1256	29.65	
.1990							11.53
Average.			63.725	6.305			

Calculated for $C_{17}H_{20}O_6$: carbon, 63.74 per cent; hydrogen, 6.25 per cent.
Found..... carbon, 63.72 per cent; hydrogen, 6.30 per cent.

Calculated for Ba ($C_{17}H_{18}O_6$): barium, 29.15 per cent.
Found..... barium, 30.09 per cent.

A molecular weight determination by the elevation of the boiling point in chloroform solution gave the results shown in Table VI.

TABLE VI.—*Ebullioscopic determination of the molecular weight of mycophenolic acid.*

Weight of substance.	Weight of solvent.	Rise of boiling point.	Molecular weight.
Gram.	*Grams.*	*Degree C.*	
0.1641	30.32	0.065	308
.1578	30.32	.060	321
Average.			314.5

Molecular weight calculated for $C_{17}H_{20}O_6$..... 320
Molecular weight found from titration..... 345.4
Molecular weight found from barium content of salt..... 328
Molecular weight found from boiling-point elevation..... 314.5

The formula $C_{17}H_{20}O_6$ may therefore be assigned to mycophenolic acid. It does not readily decompose carbonates at ordinary temperatures. It is apparently a dibasic acid, or, at any rate, combines with two atoms of a monovalent base. Whether the base combines entirely with carboxyl groups or with phenol groups has not been determined.

The acid seems to form two series of salts. Presumptive evidence on this point was obtained by the following experiments: Two decigrams of free acid were suspended in water and one equivalent of potassium hydroxid added. Unfortunately, this quantity was not sufficient to dissolve the substances completely, so that a slightly greater quantity of the alkali had to be used. This solution was then treated with one equivalent of barium chlorid. On standing in the desiccator a crystalline barium salt formed. This salt was evidently different from the normal barium salt, which is so insoluble that it precipitates at once. It was also of different appearance under the microscope, consisting of a few small needles in clusters, which appar-

ently were the normal salt, and more abundant larger single needles, apparently the acid salt. The presence of the normal salt in small quantities under the conditions of the experiment was probably due to the fact that an excess of alkali had to be used in dissolving the substances. The barium content of this preparation was determined, 0.207 gram yielding 0.0692 gram of $BaSO_4$, equivalent to a barium content of 20.2 per cent.

Calculated for Ba ($C_{17}H_{18}O_6$)	28.1 per cent.
Calculated for Ba ($C_{17}H_{19}O_6)_2$	17.7 per cent.
Found	20.2 per cent.

Apparently, as shown by the microscope, the preparation consisted of a mixture of two salts.

It has not been found possible to identify mycophenolic acid with any known compound. The substance of Gosio referred to above very greatly resembles it, though these substances are probably not identical. However, Gosio's characterization of this substance was based on a very small quantity of material, so that his formula $C_9H_{10}O_3$, based on a single combustion, can not be regarded as final. The chief points of difference between the substance described by Gosio and mycophenolic acid are the percentage composition and the behavior with ferric chlorid. Gosio's substance gives an intense blue color with ferric chlorid in alcoholic solution. Mycophenolic acid gives a violet color in aqueous solution, while in alcoholic solution with a trace of ferric chlorid it gives a violet color which becomes bright green on addition of an excess of the reagent.

In one particular mycophenolic acid resembles Gosio's substance but differs from penicillic acid. It is not toxic. Ten milligrams were dissolved in water with the aid of a little sodium carbonate and injected subcutaneously into a mouse. No untoward effects whatever were noted. It differs furthermore from penicillic acid in being present chiefly in the mycelium in the early stages of growth. In old cultures it is found both in the culture fluid and in the mycelium, perhaps because with the gradual production of basic substances it is dissolved. The question whether toxic phenolic substances are found in the culture fluid or only in the mycelium is one that has been much discussed by students of pellagra. When the substances are insoluble acids with soluble salts like mycophenolic acid, their distribution is probably only a question of the reaction of the medium. When the reaction is acid they will be found in the mycelium, as lichen acids incrust the lichen thallus. When the medium contains available bases they will become more or less dissolved in the medium.

With the advancing age of the culture, mycophenolic acid gradually increases in quantity until under the conditions employed in these experiments at the end of two weeks the maximum yield is obtained.

After that time the quantity present is apparently constant. When grown in the "Long Blake" bottles charged with 250 cubic centimeters of culture fluid, the yield at the end of about two weeks averages about 0.07 gram of the crude acid per bottle.

Since *Penicillium stoloniferum* is found so commonly in the United States and has been isolated in this laboratory from spoiled maize, it is not easy to understand why it so rarely, if ever, causes spoiled maize in the United States to give the ferric-chlorid reaction. The first explanation to present itself was that the American organism might be a different strain or perhaps a "physiological variety."

To solve this question, Dr. Thom very kindly furnished a specimen of his type culture. This specimen was grown side by side with the Italian organism. It grew rather more slowly than the latter and there were slight differences in appearance. The cultures gave a good ferric-chlorid reaction, very similar in shade to that given by the Italian organism, but when the attempt was made to separate mycophenolic acid from the cultures of the American organism none could be found. In its place was found a quite different substance or mixture of substances. As this material has not yet been obtained in satisfactory crystalline form, not much can at present be said of its properties.

The different biochemical behavior of the two strains from the two continents is certainly suggestive. Whether these two strains are really physiologically different can not as yet be decided. The American organism used is an old one, having been propagated by Dr. Thom in the laboratory for a number of years. Possibly this long artificial propagation has altered its behavior. It is proposed to continue the investigation of this problem by comparing the two cultures on hand with a number of other recently isolated strains.

No extended physiological studies were undertaken on *Penicillium stoloniferum*. A few observations were made incidentally. The organism always produced alcohol, as shown by applying the iodoform test to the distillate. No quantitative determinations were made, but the amount of alcohol formed, as judged by the iodoform test, seemed to be decidedly less than that produced by *P. puberulum*. *P. stoloniferum* produces a small amount of oxalic acid, as shown by the method used for *P. puberulum*. It seems to be present in somewhat larger amounts and at an earlier stage of growth than in cultures of *P. puberulum*. Finally the mycelium of *P. stoloniferum* seems to be very rich in mannitol.

SUMMARY.

Of six species of Penicillium from maize examined, only two elaborated substances toxic to mice. Two of these species, one toxic, the other nontoxic, were studied in detail.

The first, identified as *Penicillium puberulum* Bainier, elaborates a toxic product which was isolated and for which the name "penicillic acid" and the formula $C_8H_{10}O_4$ are suggested. This substance behaves like a monobasic acid. It is toxic to animals when injected subcutaneously, causing death in a dosage of about 0.2 to 0.3 gram per kilo of body weight. The formation of penicillic acid is more abundant when the air supply is limited and the reaction of the medium is acid. The form in which nitrogen was offered the fungus seems also to have some influence on its formation.

Penicillium puberulum Bainier was always found to produce alcohol when grown in the presence of sugar. Old cultures contain minute amounts of oxalic acid. In the presence of sugar and leucin no amyl alcohol is produced, although leucin is consumed. A small quantity of volatile acid is, however, formed. In the presence of sugar and tyrosin neither tyrol nor tyrosol is produced, though tyrosin is consumed. A small quantity of volatile acid is formed.

The second organism, *Penicillium stoloniferum* Thom, was nontoxic. Unlike the other five studied, it was isolated from Italian maize. It elaborates a new phenolic acid, for which the name mycophenolic acid and the formula $C_{17}H_{20}O_6$ are suggested. This substance behaves like a weak dibasic acid and, like penicillic acid, resembles the lichen acids in many ways. Among the other metabolic products of the organism, alcohol, oxalic acid, and mannitol were found.

In the present paper it has been shown that species of Penicillium so closely related that until recently they were not distinguished by morphologists differ quite markedly in their metabolism. It is greatly to be desired that the whole genus be studied biochemically. The chemical findings will no doubt supplement the morphological in many important ways. Indeed, as indicated by the constant presence of alcohol and the formation of penicillic acid by *Penicillium puberulum* and the formation of mycophenolic acid by *P. stoloniferum*, it is not impossible that characteristic chemical properties may help to distinguish between species or strains not now sharply separated by morphologists.

Therefore the present investigation furnishes additional data for explaining the discrepancies in different biochemical investigations on molds. Previous investigators may have failed to realize that the products elaborated vary with the species, with the reaction of the medium, with the aeration, and perhaps with the nature of the nitrogenous food supply, and that it is exceedingly difficult to distinguish between quite distinct species. They may also have underestimated the difficulties of distinguishing between the individual species.